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Identification of host-bacteria interactions following Streptococcus gordonii bacteraemia

Identification of host-bacteria interactions following *Streptococcus gordonii* bacteraemia

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Abstract

Infective endocarditis (IE) is a life-threatening disease, associated with an accumulation of bacteria and host components as vegetations on the endocardial surface of the heart. *Streptococcus gordonii*, an initial coloniser of the oral cavity, is frequently associated with IE pathogenesis. Previous studies have indicated the capacity for *S. gordonii* to interact with platelets within the blood to promote vegetation formation, mediated by surface adhesins PadA and Hsa. However, few studies have explored the ability of this bacterium to survive upon entry into the blood stream or to interact with cardiac tissue. The aim of this project was therefore to better characterise the interactions of *S. gordonii* with blood components and human coronary artery endothelial cells (HCAEC), with a particular focus on the roles of PadA and Hsa. Proteomic studies were used to identify interactions of PadA/Hsa with HCAEC and plasma proteins, combined with in vitro assays to investigate survival of wild-type *S. gordonii* or PadA/Hsa knockout mutants in human serum and their interactions with vitronectin and neutrophils. Potential strategies were identified that may enable *S. gordonii* to evade complement-mediated killing and other immune defences, to which both Hsa and PadA contributed. Further insight into the capacity for *S. gordonii* to target damaged cardiac tissue and exacerbate vegetation formation was also provided. Taken together, this information advances understanding of the mechanisms that may enable *S. gordonii* to survive within blood and promote IE which, in turn, could assist the development of novel treatment approaches to combat IE.

Acknowledgements

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Finally, a big thank you to my family, friends, and boyfriend who have all helped me throughout this difficult and sometimes emotional year, you've all been great and have all kept me afloat when things did not go to plan!

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of Abbreviations

BHY	brain heart infusion broth plus yeast extract
BHYN	BHY plus neopeptone
BSA	bovine serum albumin
CFU	colony forming units
CSP	competence stimulating peptide
dH_2O	distilled water
EPS	extracellular polymeric substance
FH	Factor H
FH-L	Factor H-like protein
GAS	group A streptococci
h	hour
HCAEC	human coronary artery endothelial cell
HOMD	human oral microbiome database
Hsa	haemagglutinin salivary antigen
IE	infective endocarditis
IL	interleukin
MAC	membrane attack complex
min	minute
NETs	neutrophil extracellular traps
NOD	nucleotide oligomerization domain
PadA	platelet adherence protein A
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PHOX	NADPH oxidase enzyme complex
PPP	platelet rich plasma
PRP	platelet poor plasma
ROS	reactive oxide species
s	second
SD	standard deviation
SndA	streptococcal nuclease domain A
SRR	serine-rich repeat
SWAN	streptococcal wall-associated nuclease
TLR	Toll-like receptor

TraDIS	transposon directed insertion site sequencing
WBC	white blood cell
ZmpC	zinc metalloproteinase C

COVID-19 Impact Statement

Unfortunately, the UK went into national lockdown at the end of March 2020, meaning that all postgraduate laboratory work had to be discontinued within the university. A visit to the Waller group to complete TraDIS studies was planned for April 2020, but this had to be cancelled due to the circumstances and the studies described in this project were therefore halted. In addition, further work was planned for this project throughout the summer months, including the use of flow-cytometry and further experiments using vitronectin which unfortunately could not be carried out due to UK lockdown. Throughout this project, there were several difficulties faced, such as the breakdown of laboratory freezer meaning that samples were lost, and incorrectly prepared samples which were to be used for experiments. Some of the *S. gordonii* surface protein experiments would have included both single and double knock-out strains, however, these overnight cultures were incorrectly prepared or were prepared using thawed reagents, rendering these cultures unsuitable for experimental work right at the last minute, before lockdown began.

In order to complete the project to a high standard, proteomic analysis was included, where previous generated data was analysed further to identify interactions that were not observed in previous studies. Because TraDIS studies and further work using blood could not continue, proteomics studies could be completed from home and over online calls to the proteomics facility. Proteomics studies would then become a large part of this thesis, replacing experiments that would have been carried out if lockdown hadn't happened.

Chapter 1

Introduction

1.1 The oral environment

It is estimated that around 700 different prokaryotic species are able to inhabit the oral cavity, with those identified to date recorded in the Human Oral Microbiome Database (HOMD). Up to 200 of these species may be found in any one individual at a given time (Cunha et al., 2010). Around 13% of these species are unnamed, and it has been challenging to fully characterise every species due to, for example, cultivation difficulties. However, some of the most abundant species found within the oral cavity are those belonging to the *Streptococcus* genus, with around 26 species found able to inhabit the oral cavity to date (Cunha et al., 2010).

The oral cavity contains a range of different microenvironments and habitats for these prokaryotes; for example, hard tissue surfaces such as the teeth and softer tissues such as the gingival sulcus, tonsils, tongue and gingivae (Cunha et al., 2010). These environments can be hostile and difficult to survive in, due to the constant movement of food and the tongue, oral hygiene procedures carried out by the host such as brushing or flossing, and the release of acids into the environment by some commensal species within the oral cavity in a bid to out-compete others (Cunha et al., 2010). To overcome these conditions, many bacterial species within the oral cavity form a biofilm to allow them to survive.

A biofilm is a collection of microorganisms surrounded by extracellular polymeric substances (EPS), which can provide the microorganisms with protection, nutrients and oxygen availability (Cunha et al., 2010). Dental plaque is an example of a biofilm, composed primarily of oral microorganisms, components of food such as sugars, EPS and salivary components, which in turn are bound to the salivary pellicle on teeth (Huang et al., 2011). This salivary pellicle is a thin, acellular film composed of salivary proteins and glycoproteins that coats all surfaces of the oral

cavity. Several oral bacteria, such as *Streptococcus gordonii* and *Streptococcus mutans*, are able to adhere to and subsequently colonise the pellicle found on teeth, which can initiate formation of the plaque biofilm (Rath et al., 2017). These bacteria are often referred to as primary colonisers.

As shown in Figure 1.1, bacteria first entering the oral cavity will be present in a planktonic state, which allows them to freely move within saliva. Primary colonisers will interact with and bind to the salivary pellicle. These bacterial cells can then co-adhere with other cells, allowing further microorganisms to be recruited to the growing biofilm by attaching to those cells already bound. Ultimately, all of these species exist as a community in a 'sessile' or static state, which is embedded within a matrix of extracellular polymeric substances (EPS) produced by the microorganisms. This represents the mature biofilm stage, and microbes may later undergo dispersion and release from the biofilm (Huang et al., 2011).

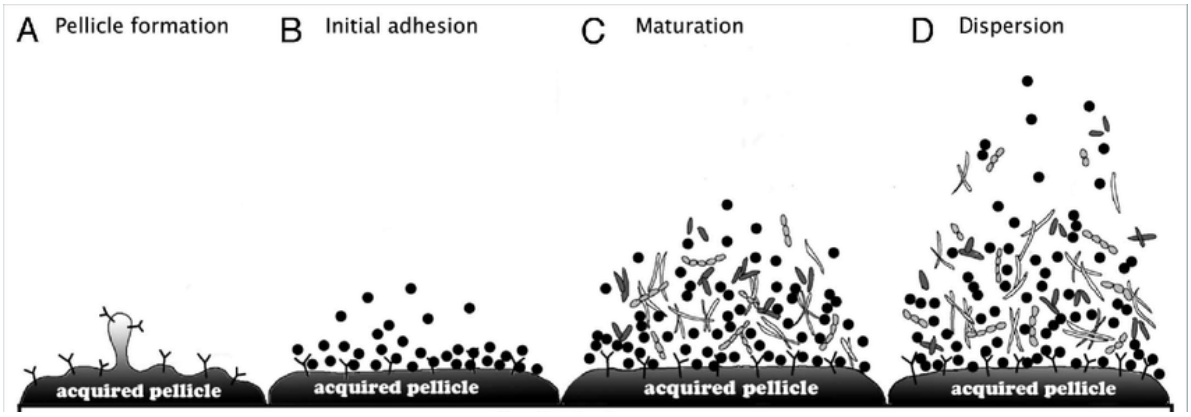


Figure 1.1 – Simplified schematic to illustrate dental plaque biofilm formation

A – Formation of the salivary pellicle on the tooth surface. B – Initial adhesion of primary colonisers, such as oral streptococci, to the salivary pellicle. C – Maturation of the biofilm as more species join via co-adhesion and produce EPS, entering a sessile state. D - Dispersion of the oral biofilm. Reproduced from Huang et al. (2011).

1.2 Oral health vs. general health

Oral disease is a major area of research focus from both an oral biology and public health perspective. Oral diseases such as periodontitis are some of the most prevalent, chronic health conditions seen around the world (Griffin et al., 2012) and, if left untreated, can lead to prolonged pain and adverse health outcomes (Abranches et al., 2018). The health of the oral cavity can significantly impact an individual's ability to eat and make food choices, with studies showing that tooth loss amongst older people can contribute to weight loss, obesity or vitamin depletion. An individual with tooth loss due to oral disease will likely choose easier-to-chew foods, due to their lack of complete dentition or pain experienced during eating (Savoca et al., 2010). Additionally, tooth loss can have an emotional impact, and be detrimental to an individual's self-esteem and well-being. Studies have demonstrated that individuals with tooth loss are more likely to avoid social situations or eating in public, for fear of embarrassment or wishing to hide their appearance (Gerritsen et al., 2010). Oral disease therefore contributes to several problems an individual may face, not only including pain and difficulty eating, but negatively impacting an individual's self-confidence.

Alongside local disease manifestations, it has been widely recognised for several decades that there is a relationship between oral health and general health. It has been suggested that the oral cavity is a 'window to general health', at the intersection of medicine and dentistry, as it is an area of the body that interacts with the external environment. It is also the entry point for both the respiratory and gastrointestinal tracts within the body, a feature that can be both harmful and beneficial for the health of the individual (Gomaa et al., 2016). Early mentions of the link between oral health

and systemic disease date back to when Hippocrates claimed to have cured a patient's rheumatoid arthritis by removing one of their infected teeth (Kumar, 2017). In the 17th century, a Dutch scientist, Antonie van Leeuwenhoek, obtained a sample of his own dental plaque by scraping his teeth to test his new microscope. He famously described what he saw on the glass slide as 'little animalcules', introducing the idea that the oral cavity contained small, living microorganisms that were likely causing disease within the oral cavity and throughout the body (Chapple, 2009). This discovery, alongside Hippocrates' claims, eventually contributed to the introduction of the 'focal infection theory' in the 19th and 20th centuries, which suggested that oral infections may be responsible for other diseases found around the body. This led to medics at the time hypothesising that infections that are localised at one site may eventually spread to other parts of the body (Chapple, 2009).

Based on the focal infection theory, in more recent centuries the removal of diseased teeth has been commonplace in dental practice to minimise risk of systemic disease, especially as a strategy to avoid cardiac disease or rheumatoid arthritis (Kumar, 2017). Today, such procedures are now driven by an even greater understanding of the relationship between oral disease and general health. For example, prior to a patient undergoing heart surgery, an oral clearance examination is necessary to assess whether poor oral health contributed to the heart issue or could contribute to further infection post-surgery. In addition, any infected teeth are often removed prior to surgery, to ensure they do not contribute to further infection or healing issues (de Souza et al., 2016).

1.3 Oral streptococci in health and disease

Streptococci are Gram-positive, non-motile, non-spore forming facultative anaerobes that commonly grow in pairs or chains. Streptococci are characteristically round or ovoid in shape and approximately 1 μm in diameter (Yumoto et al., 2019). They are found in association with mucosal tissues throughout the human body, including the upper respiratory tract, and account for approximately 20-30% of all bacterial species within the mouth, although this can vary amongst individuals (Cahill et al., 2017). Streptococci were initially classified based on their ability to undergo haemolysis on blood agar, with β-haemolysis meaning complete haemolysis, α-haemolysis meaning partial haemolysis and γ-haemolysis meaning non-haemolytic. Species that undergo α-haemolysis on blood agar are referred to as 'viridans' streptococci, taken from the Latin word *viridus*, which means green (Abranches et al., 2018). These bacteria produce hydrogen peroxide, which oxidises haemoglobin within blood to green methaemoglobin, and a number of oral streptococci fall within this group (Facklam, 2002). Viridans streptococci are associated with a number of systemic infections and are commonly isolated from patients with bacterial endocarditis or from neutropenic patients suffering from cancer. Viridans streptococci can be difficult to eradicate using antibiotics, and penicillin resistance was shown to be as high as 48% in strains obtained from the USA (Facklam, 2002).

A modern approach for classifying streptococci is based on 16S rRNA sequencing, and this has resulted in the oral streptococci being divided into six major phylogenetic groups: anginosus, mitis, sanguinis, salivarius, downei and mutans groups (Richards et al., 2014). The mitis and sanguinis groups are commonly associated with systemic infection in adults, and bacterial species within these

groups, such as *S. gordonii*, are frequently isolated from patients with infective endocarditis (IE) and in rare cases, with bacterial meningitis (Cahill et al., 2017; Kutlu et al., 2008).

1.3.1 Role in oral health

Oral streptococci, such as members of the mitis, sanguinis and mutans groups, have been traditionally considered as 'commensals' within the oral cavity, but with improved understanding of the delicate and dynamic interplay between members of the oral microbiota, this description is now considered too restrictive. Many oral streptococci can act as probionts, which can be beneficial for the health of the host. One example is *S. gordonii*, which can promote oral health by neutralising acids produced by *S. mutans* (Krantz et al., 2017). Most oral streptococci also produce hydrogen peroxide (H_2O_2), which can inhibit *S. mutans* and *Porphyromonas gingivalis*. This is advantageous to the host, as *S. mutans* is strongly associated with dental caries, while *P. gingivalis* is frequently isolated from sites of aggressive periodontitis (Abranches et al., 2018). Therefore, the presence of oral streptococci can act to dampen the effects of pathogenic species, thus preventing irritation and inflammation by the host immune system. In contrast, some oral streptococcal species may also serve as 'accessory pathogens' (Lamont et al., 2018), based on their capacity to enhance disease progression when in association with other pathogenic microbes. For example, using a mouse model of periodontal disease, alveolar bone loss was enhanced for *P. gingivalis* in the presence of *S. gordonii* compared to *P. gingivalis* alone. Therefore, in recognition of their association with both oral health and disease, species such as *S. gordonii* have also been designated as 'opportunistic' pathogens (Cahill et al., 2017).

1.3.2 Role in systemic disease

Upon entering the bloodstream, some oral streptococci have been shown to contribute to or cause a number of systemic infections (Yumoto et al., 2019). One such example is septic arthritis. This is an infection around artificial joints that can be exacerbated or caused by oral streptococci and usually occurs when a mixed infection of skin commensal species, such as *Staphylococcus aureus*, occurs alongside oral bacteria that have entered the blood. Surgical cultures have been obtained from an infected artificial joint replacement and found to contain oral *S. gordonii* (Klein et al., 2015).

Disseminated intravascular coagulation (DIC) is another bloodstream-related condition that has been linked with oral streptococci. DIC is characterised by the formation of blood clots throughout the circulatory system, which may result in organ failure or death. This can often be triggered by bacterial or fungal infections within the blood and group A *Streptococcus* (GAS) has frequently been associated with DIC, in particular during pregnancy (Bryant, 2003). It has been suggested that oral streptococci, such as *S. gordonii*, which are capable of inducing platelet activation and subsequent thrombus formation, may play a role in DIC due to the enhanced clotting of platelets within the bloodstream (Nobbs et al., 2009).

Aspiration of oral bacteria into the lungs can cause systemic disease in some patients (Cahill et al., 2017). Pneumonia can result from infection by oral streptococci, especially amongst patients who suffer from breathing or swallowing difficulties. In a study of 177 pneumonia patients, analysis of 16S rRNA within

patient bronchioalveolar lavage fluid identified *S. mitis* and *S. oralis* as present, in addition to *S. pneumoniae* (Akata et al., 2016).

Oral streptococci are also associated with IE, which is discussed in further depth in section 1.5.

1.4 Role of streptococcal surface proteins in colonisation and pathogenesis

In general, to be able to colonise the human host or cause infection, bacteria must first be able to adhere to host tissues, and this is often facilitated by surface proteins, known as adhesins (Chapple, 2009). Oral streptococci express a variety of adhesins which, in turn, confer the ability to bind a range of substrata at both oral and extraoral sites. For example, oral streptococci can attach to components of the extracellular matrix (ECM), such as fibronectin (Back et al., 2017), and components of blood, such as platelets and neutrophils (Haworth et al., 2017a; Urano-Tashiro et al., 2008). The adhesins found on oral streptococci can be grouped based on their structure, but the majority are anchored to the cell wall by transpeptidase sortase A via a C-terminal LPxTG motif (Nobbs et al., 2009). Table 1.1 summarises some of the key streptococcal LPxTG surface protein families found in oral streptococci.

Table 1.1 LPxTG surface protein families found in oral streptococci

Protein family	Example protein	Species	Function	Reference
Ag I/II family	SspA/SspB SpaP	*S. gordonii* *S. mutans*	Coaggregation; adherence to multiple substrata i.e. epithelial cells and collagen	(Jakubovics et al., 2009; Matsumoto-Nakano, 2018)
Serine-rich repeat family	Hsa SrpA	*S. gordonii* *S. sanguinis*	Adherence to multiple substrata i.e. platelets	(Deng et al., 2014; Haworth et al., 2017a)
Pili and fibrils	CshA/CshB PilA/PilB/PilC	*S. gordonii* *S. sanguinis*	Coaggregation; adherence to multiple substrata; biofilm formation	(McNab et al., 1996; Okahashi et al., 2010)
Other LPxTG proteins	PadA	*S. gordonii*	Binding to platelets; binding to ECM	(Haworth et al., 2017b; Jakubovics et al., 2009)
	SWAN SndA	*S. sanguinis* *S. gordonii*	DNA degradation i.e. neutrophil extracellular traps	(Back et al., 2017; Morita et al., 2014)

1.4.1 AgI/II polypeptides

The antigen I/II (AgI/II) family polypeptides are found in most species of oral streptococci, and this was one of the first adhesin families to be discovered in Gram-positive bacteria. These polypeptides play a key role in biofilm formation, platelet aggregation and tissue invasion, particularly during the onset of periodontal and cardiovascular disease (Brady et al., 2010). Within the oral cavity, AgI/II proteins are essential for species such as *S. mutans* and *S. gordonii* to attach to the salivary pellicle, thus preventing their removal from the oral cavity via the flushing action of saliva (Jakubovics et al., 2005; Petersen et al., 2002).

AgI/II family polypeptides have a defined domain structure, including blocks of alanine-rich repeats and proline-rich repeats that flank a central variable region (Figure 1.2). *S. gordonii* expresses two AgI/II family polypeptides, designated SspA and SspB, which contribute both to coaggregation with other bacteria and adherence to multiple substrata such as salivary glycoprotein-340 and epithelial cells (Brady et al., 2010; Nobbs et al., 2009).

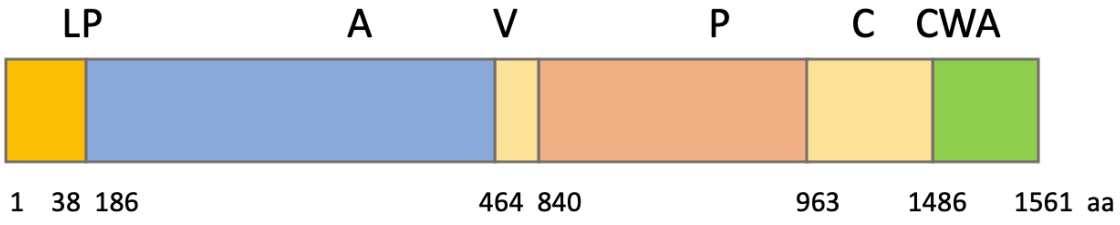


Figure 1.2 – Structural features of an antigen I/II family polypeptide. Schematic indicates a leader peptide (LP), alanine-rich repeats (A), a variable region (V), proline-rich repeats (P), a C-terminal region (C), and cell-wall anchorage region (CWA). Reproduced from Brady et al. (2010).

1.4.2 SRR family

The serine-rich repeat (SRR) family of polypeptides are glycosylated and contain blocks of serine-rich amino acid residues. The number of amino acid residues within each SRR protein is highly variable, thus they all differ in size (Lizcano et al., 2012). A key SRR protein found on the surface of *S. gordonii* DL1 is haemagglutinin salivary antigen (Hsa), which binds to sialylated carbohydrate regions found on multiple targets such as salivary glycoprotein-340 (gp-340) (Takamatsu et al., 2005). In fact, studies have shown that adherence of *S. gordonii* DL1 to surface-bound gp340 is dependent upon the expression of Hsa (Loimaranta et al., 2005). In addition to glycoproteins, Hsa plays an important role in the binding of bacterial cells to platelets via platelet membrane glycoprotein Ibα, especially during shear-enhanced adhesion. During this mechanism, the shear-flow of blood over immobilised bacterial cells enhances the adherence between bacteria and platelets. As the blood flow increases, platelet rolling slows to allow for firm adhesion to the bacterial cell surface (Yakovenko et al., 2018).

Hsa contains an N-terminal signal peptide, a basic amino acid residue region (BR), two serine-rich regions (SRR1/2) and a C-terminal cell-wall anchoring domain (Figure 1.3). The BR has been shown to contribute to the adhesion of Hsa and may be a determinant in the functional differences between homologous SRR proteins (Takamatsu et al., 2005)

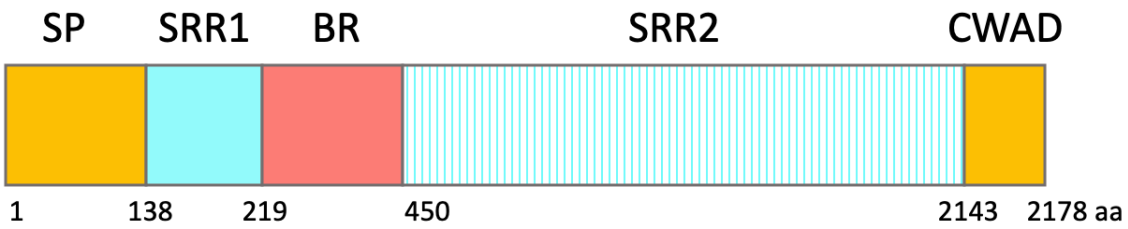


Figure 1.3 – Structural features of the Hsa protein found in *S. gordonii* DL1 (Challis). Schematic indicates signal peptide (SP), serine-rich repeat regions (SRR1/2), basic amino acid residue region (BR) and cell wall anchoring domain (CWAD). Reproduced from Nobbs et al. (2009).

1.4.3 Pili/fimbriae

Several oral streptococci possess filamentous structures known as fibrils or pili (Nobbs et al., 2009). Fibrils are flexible rods approximately 40-400 nm in length, whereas pili are 0.3-1 nm. The CshA protein of *S. gordonii* was identified as the main structural component of one of its fibrils and mediates coaggregation with other oral bacteria and attachment to fibronectin (McNab et al., 1996). The binding of bacteria to fibronectin promotes bacterial colonisation at multiple sites throughout the host, such as oral epithelia or cardiac tissue in the case of *S. gordonii*. Adherence of *S. gordonii* to cardiac endothelium via fibronectin bridging is one potential mechanism that may promote the development of IE (Back et al., 2017). CshA contains a 3-domain non-repeat region and basic amino acid repeat region (Figure 1.4) (Back et al., 2017). Similar fibrils have been found on the surfaces of *S. sanguinis* and *S. oralis* (Back et al., 2017; Nobbs et al., 2009).

Pili are not as common on the surface of oral streptococci but have been reported for *S. sanguinis* (Zhu et al., 2018). Unlike fibrils, pili are formed by the covalent attachment of multiple protein subunits. In *S. sanguinis*, pili comprise PilA, PilB and PilC proteins, which can facilitate attachment to fibronectin and other components of the ECM (Okahashi et al., 2010).

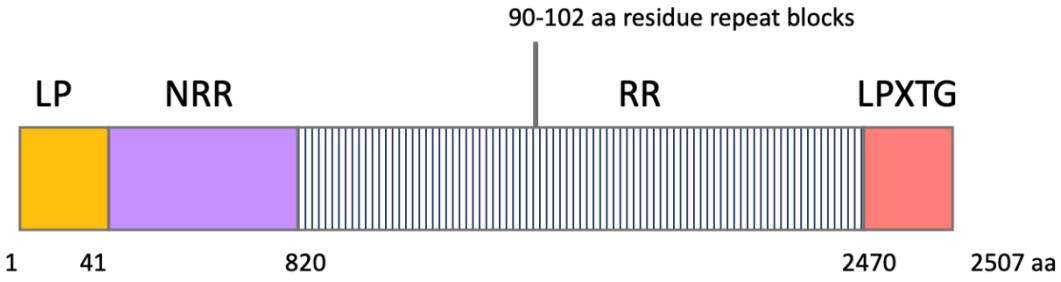


Figure 1.4 - Structural features of the CshA protein found in *S. gordonii* DL1 (Challis). Schematic indicates leader peptide, a 3-domain non-repeat region (NRR), an amino acid repeat region (RR) and LPxTG C-terminal anchor motif. Reproduced from Back et al. (2017).

1.4.4 PadA

Platelet adherence protein A (PadA) is another important surface protein found in *S. gordonii*. PadA facilitates the interactions of *S. gordonii* with platelets by targeting platelet receptor GPIIbIIIa, which is considered critical to the capacity for *S. gordonii* to promote IE (Petersen et al., 2010). Previous studies have shown that the PadA N-terminal region mediates interactions with platelets (Keane et al., 2010), but more recently this region was also found to bind vitronectin, fibronectin and salivary pellicle (Haworth et al., 2017a). PadA therefore plays an important role in facilitating

colonisation of the oral cavity by contributing to the ability of *S. gordonii* to form biofilms on oral surfaces, such as the teeth and epithelia.

PadA contains an N-terminal F2 region, comprising a von Willebrand Factor-like domain, NGR, RGT and AGD motifs, followed by an extensive repeat region of amino acid residues (Figure 1.5) (Haworth et al., 2017b).

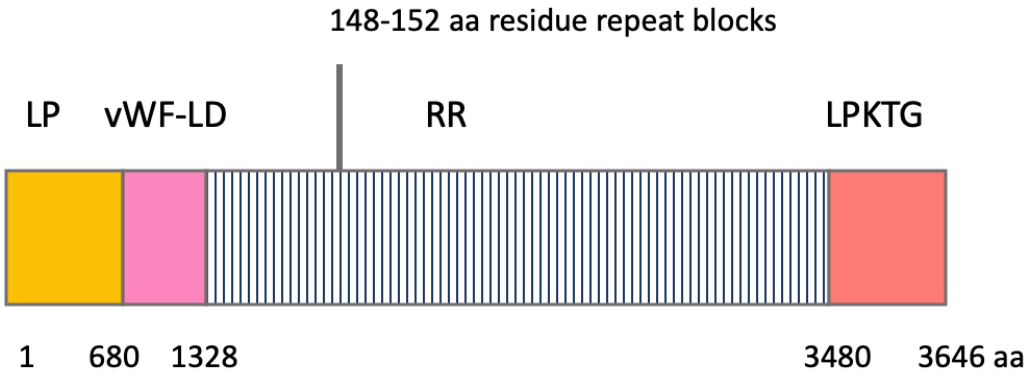


Figure 1.5 – Structural features of the PadA protein found in *S. gordonii* DL1 (Challis). Schematic indicates N-terminal region, F2 region comprising a von Willebrand factor-like domain (vWF-LD) and NGR, RGT and AGD motifs, repeat region of amino acid residues and C-terminal LPKTG cell-wall anchor motif. Reproduced from Haworth et al. (2017)

1.4.5 Cell-surface nuclease

Oral *S. sanguinis* expresses a surface-bound nuclease, known as streptococcal-wall anchored nuclease (SWAN). This nuclease is capable of degrading DNA and has been shown to play a role in *S. sanguinis* evasion of neutrophil extracellular traps (NETs) by degrading the NET DNA structure (Morita et al., 2014). This

nuclease is homologous to streptococcal nuclease domain A (SndA) found on *S. gordonii,* which also exhibits DNase activity (unpublished data).

1.5 Infective endocarditis

One systemic disease with which oral streptococci have been particularly associated is IE. IE is an umbrella term for the infection of a native or prosthetic heart valve or endocardial surface by circulating bacteria (Cahill et al., 2017). IE can also include bacterial infection of an indwelling cardiac device, such as a pacemaker (Holland et al., 2016). Typically, clots of bacteria, platelets and immune cells will form on the cardiac tissue, commonly known as septic or infective thrombi or vegetations. This can lead to a septic pulmonary embolism, which can be life threatening (Holland et al., 2016).

Diagnosing IE is difficult and time-consuming, which can allow the disease to progress and lead to irreparable valvular damage, which often requires invasive surgery. The optimal therapeutic strategy varies from patient to patient, but antibiotic or anticoagulant therapy is often not successful due to antibiotic resistance or difficulty with the agent penetrating vegetations (Gould et al., 2012; Thuny et al., 2012).

1.5.1 Epidemiology

Over the past five decades, the incidence of IE has been relatively rare, and the prevalence of the disease shows significant variation across continents (Li et al., 2000). In low-income countries with poorer access to healthcare and sanitation,

rheumatic heart disease has remained the key risk factor for IE, particularly amongst young adults and men (Cahill et al., 2017). Additional risk factors worldwide include both intravenous drug use and diabetes, as these can facilitate direct access to the blood by skin commensals via needles or open wounds and ulcers (Thuny et al., 2012). In higher-income countries such as the UK, a number of risk factors for IE are associated with advancements in medical treatments (Cahill et al., 2017). Invasive procedures such as valve replacement and indwelling cardiac devices amongst a variety of age-groups are major contributors to IE development. This is due to the level of open-heart surgery required, which increases the likelihood of infection both during and after surgery (Baddour & Prendergast, 2018).

1.5.2 Pathophysiology

In health, the valvular endothelium and cardiovascular system should be a sterile environment, resistant to microbial colonisation (Mathew & Bhimji, 2018). However, microorganisms may enter the bloodstream by a variety of routes, leading to bacteraemia. Common routes of infection are by contamination of oral or skin wounds, or infection of indwelling medical devices such as catheters or intravenous lines (Baddour et al., 2015). In recent years, oral disease or trauma caused by, for example, tooth extraction or periodontal surgery, have been recognised as contributing factors to IE development (Cahill et al., 2017). Daily events such as mastication (chewing) also have potential to facilitate the transient entry of bacteria into the blood (de Souza et al., 2016).

Once bacteria are in the blood, they utilise a variety of virulence factors to enable survival within the hostile and often turbulent environment of the bloodstream. These

include surface proteins that can facilitate adhesion to ECM components such as fibronectin, which can allow bacterial cells to attach and establish an initial infection site (Haworth et al., 2017a). Usually in IE patients, alongside bacteraemia, individuals will have a damaged or roughened endocardial surface. This can be caused, for example, by high blood pressure or suturing. This roughened surface provides an attachment point for viable bacteria within the bloodstream, from which the bacteria can promote thrombosis (clot formation) and the development of infective vegetations (Holland et al., 2016).

1.5.3 Microbiology

Around 80-90% of IE cases are caused by *Staphylococcus*, *Streptococcus* and *Enterococcus* bacteria, with *Staphylococcus aureus* being the most frequently isolated microorganism in high-income countries (Cahill et al., 2017). As a skin commensal, *S. aureus* often colonises indwelling lines, allowing easy access into the bloodstream. Oral viridans group streptococci are responsible for around 40-60% of endocarditis cases occurring on natural heart valves (Rath et al., 2017).

1.5.4 Mechanisms of streptococcal-induced IE

To promote IE, causative agents must be able to attach to the site of cardiac damage. For *S. gordonii*, one potential mechanism that has been proposed is via binding to exposed components of the ECM (Nobbs et al., 2009*). S. gordonii* can bind to fibronectin using its multi-fibrillar surface protein CshA, via an N-terminal repeat region. This is mediated via a ‘catch-clamp’ mechanism, which could enable

CshA to attach strongly to fibronectin, even under shear conditions (Back et al., 2017).

A key aspect of the pathogenesis of IE is then the interactions of bacteria with platelets to drive the unwanted thrombosis on heart valves. Across the oral streptococci, such interactions have been most extensively characterised for *S. gordonii*. PadA and Hsa adhesins expressed on the surface *of S. gordonii* have been shown to bind platelets in a synergistic mechanism (Haworth et al., 2017b). Hsa mediates the initial interaction with platelets via receptor GPIbα, which aids capture of platelets from the bloodstream and platelet rolling over the area of endothelial damage (Haworth et al., 2017b). As the platelets slow, PadA then interacts with the platelet receptor GPIIbIIIa, which promotes firm platelet adhesion and triggers platelet activation (Petersen et al., 2010). This, in turn, leads to further platelet recruitment due to platelet aggregation, mediated by AgI/II proteins and Hsa (Jakubovics et al., 2005). This accumulation of platelets and bacteria can be further targeted by cells of the immune system, such as neutrophils and macrophages. Ultimately, this process leads to the accumulation of host cells and bacteria, known as the septic thrombus, attached to the cardiac endothelium (Figure 1.6) (Cahill et al., 2017; Keane et al., 2010).

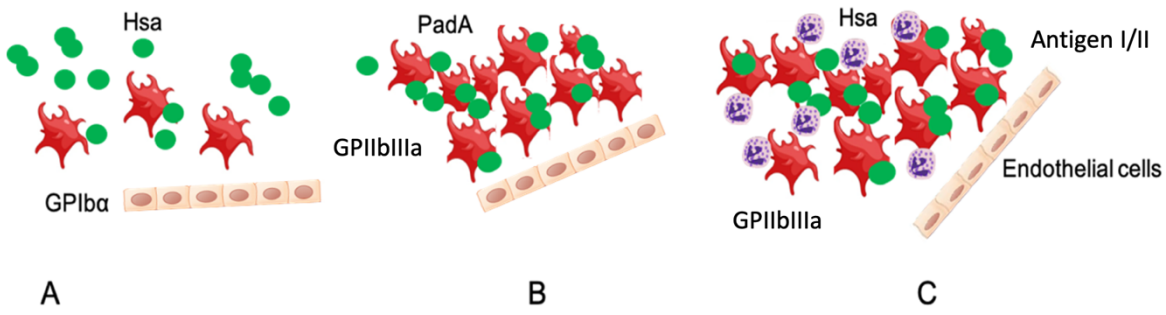


Figure 1.6 – Interactions of *S. gordonii* with platelets in IE. The initial interaction of *S. gordonii* Hsa with platelet receptor GPIbα facilitates the slowing down of platelets and platelet rolling (A). This, in turn, facilitates the interaction of *S. gordonii* PadA with platelet receptor GPIIbIIIa, leading to firm adhesion and platelet activation (B). Ultimately, additional recruitment of platelets via aggregation and interactions with immune cells leads to formation of the infective vegetation on the cardiac endothelium (C).

1.6 Bacteraemia

In health, blood is a sterile environment; however, it can become compromised by bacteria entering through a variety of mechanisms e.g. wound, surgery, toothbrushing. Once bacteria are present within blood, this is known as ‘bacteraemia’, and can cause a variety of problems and result in bacterial accumulation at vital organs (Baddour et al., 2015). Bacteraemia is a key factor in the pathogenesis of IE.

1.6.1 Components of blood

Blood is a mixture of proteins and cellular elements, which in general include platelets, erythrocytes, plasma and leukocytes (Basu & Kulkarni, 2014). Platelets regulate haemostasis and thrombosis following tissue injury (Holinstat, 2017), whilst erythrocytes facilitate the transport of gases around the body (Basu & Kulkarni, 2014). Leukocytes, commonly known as white blood cells (WBCs), circulate within the blood to help the body fight infection. They are classed into granulocytes, monocytes and lymphocytes, with granulocytes being an umbrella term for neutrophils, eosinophils and basophils (Rosales et al., 2017). The liquid component in which all of these cells are suspended is plasma. Plasma is approximately 90% water, but also contains coagulants such as fibrinogen, plasma proteins, electrolytes such as potassium and sodium, and immunoglobulins to fight infection. The term serum refers to plasma lacking fibrinogen (Mathew & Bhimji, 2018).

1.6.2 Immune defences in blood

The innate immune system is the first line of defence within the host to protect against invading microorganisms. This response is immediate and is not dependent upon previous exposure to microorganisms, in contrast to the acquired immune response. The innate immune system is comprised of many components that act in unison to protect the host against microorganisms, including physical barriers (such as mucous membranes), antimicrobial peptides (AMPs), interstitial fluid, antimicrobial proteins and WBCs (Fallis, 2013; Kobayashi et al., 2018).

The innate immune response works to rapidly recognise microorganisms and facilitate the recruitment of immune cells, such as neutrophils, monocytes,

macrophages and mast cells, to the affected area. These immune cells then function to recognise pathogen-associated molecular patterns (PAMPs) expressed by the microbes that have entered the host. Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) receptors recognise these PAMPs, amplifying inflammation and activating the adaptive immune response via antigen presentation (Carrillo et al., 2017). WBCs are the key immune cells found in blood that act to rapidly target and remove microbes, and macrophages and neutrophils are some of the first cells to arrive at a site of infection (Uriarte et al., 2016).

1.6.2.1 Neutrophils

Neutrophils mature in the bone marrow and are released into peripheral vasculature as a first line of defence during the innate immune response. Neutrophils can recognise components of pathogens or respond to inflammatory signals such as cytokines (Rosales et al., 2017). This causes neutrophils to migrate to the site of infection, utilising rolling adhesion to attach to endothelial cells (Rosales et al., 2017).

Neutrophils employ a variety of mechanisms to facilitate bacterial killing, such as the use of their granular components and phagocytosis (Figure 1.7). Neutrophils attach to bacteria using TLRs and NOD receptors, after recognition of PAMPs and opsonins on the bacterial cell surface (Kobayashi et al., 2018). Once bacteria have been recognised and bound, neutrophils begin the process of engulfing the bacterial cell, forming a phagosome. Within the phagosome, different neutrophil granule types can assemble and eventually liberate their contents, which are required for destruction of the bacteria. A key stage in bacterial killing is the production of

reactive oxygen species (ROS) and nitric oxide species (NOS) (van Kesse et al., 2014)

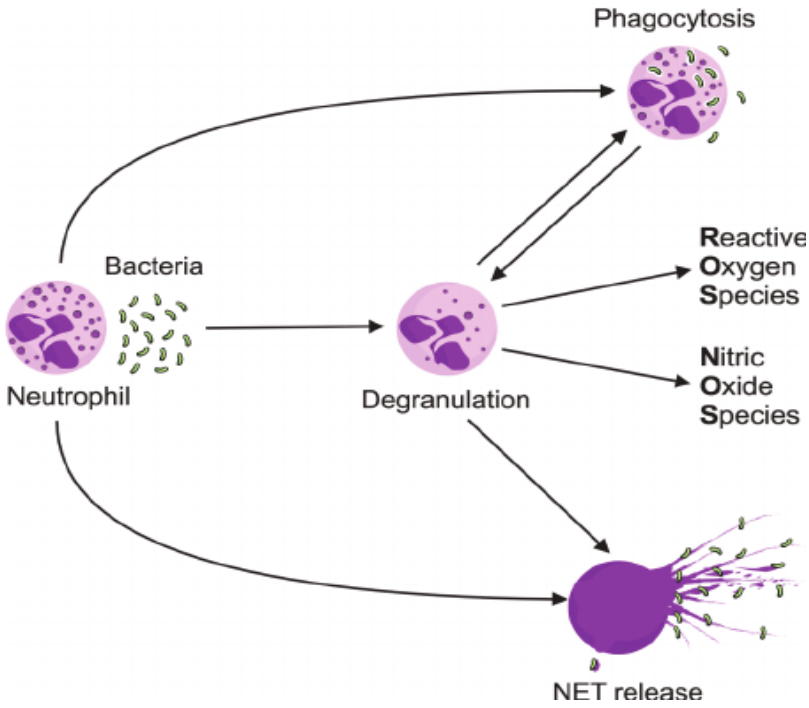


Figure 1.7 - The antibacterial actions of neutrophils once activated. Neutrophils are capable of phagocytosing bacteria or undergoing degranulation and the subsequent release of antimicrobial compounds such as ROS and NOS. Phagocytosis often requires the presence of ROS species within the phagosome to aid destruction of the bacteria. Neutrophils also release extracellular traps (NETs) during degranulation to trap and kill bacteria. Reproduced from Perobelli et al. (2015).

Another antibacterial mechanism widely employed by neutrophils is the production of NETs. NET production is often a cell death process, known as lytic NETosis, which involves the loss and expulsion of neutrophil intracellular membranes followed by plasma membrane rupture, forming a ‘net’ comprised of histone proteins and

intracellular components around the bacteria (Teng et al., 2017). ROS production is required for NETosis to occur, as activity of the NADPH oxidase enzyme complex (PHOX) is essential for autophagy of the neutrophil (Castanheira & Kubes, 2019). It is also the level of ROS present that determines whether the NETosis reaction will be lytic (ending in cell death) or non-lytic (Brinkmann & Zychlinsky, 2012; Stoiber et al., 2015).

1.6.2.2 Complement

A large component of the innate immune system is the complement system, which can be found in the blood plasma (Mathew & Bhimji, 2018). This system is comprised of approximately 40 different, inactive plasma proteins, which can be activated via various mechanisms, such as the presence of pathogen-bound antibodies (Lambris et al., 2008). There are three different pathways by which the complement system can be activated: the alternative pathway, the mannose-binding lectin pathway and the classical pathway (Figure 1.8) (Ekdahl et al., 2018). The overall outcome of the complement system is to generate a membrane attack complex (MAC), recruit phagocytes and opsonise invaded microorganisms. The MAC is a hydrophilic pore comprised of complement proteins C5-9 that spans the bacterial cell membrane, leading to membrane damage (Carrillo et al., 2017).

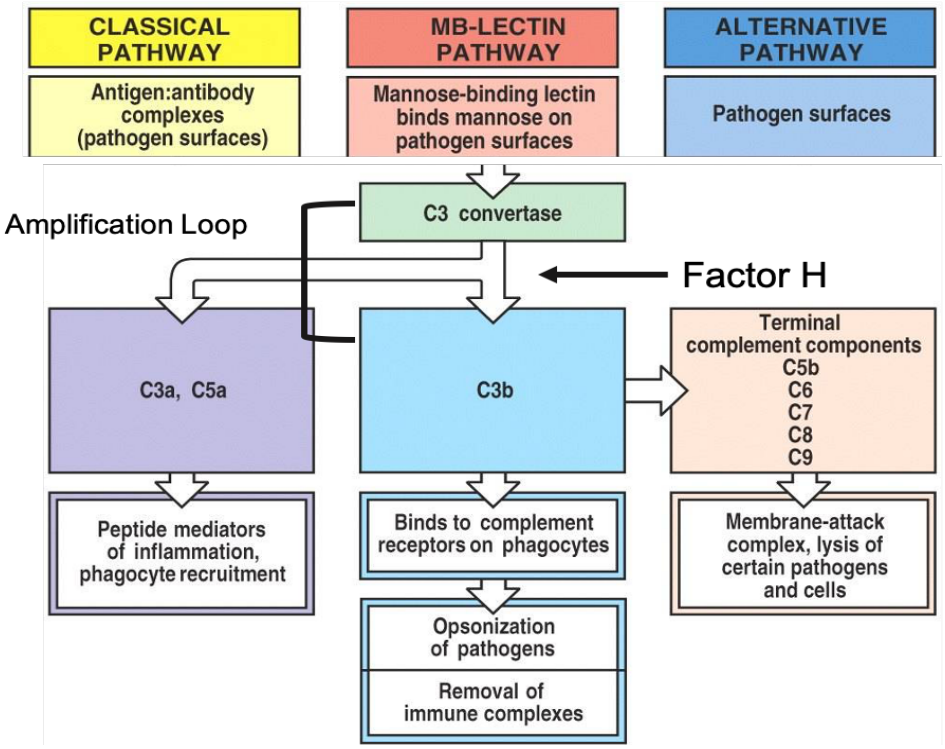


Figure 1.8 – Simplified schematic to illustrate the three complement pathways. There are three different ways in which the complement system can be activated: the classical pathway is activated primarily by antigen-antibody complexes. The C1 complex binds to the antibody, which is bound to the antigen, generating the C3 convertase enzyme complex. The mannose-binding lectin pathway is activated by mannose-binding lectins or ficolin (carbohydrate-binding proteins) to mannose groups found on the bacterial cell surface. This ultimately promotes C3 convertase activity within the classical pathway. The alternative pathway differs and is initiated by microbial surface determinants, whereby hydrolysed C3 and factor B lead to subsequent formation of the C3 convertase C3bBb. This allows further generation of the C5 convertase enzyme, which initiates formation of the C5b-9 terminal complex. This complex is known as the membrane attack complex (MAC) and is the ultimate goal of the complement system. This complex allows pores to form on the bacterial surface, which can lead to bacterial cell swelling, leakage and death. Reproduced from Janeway's Immunology (Fallis, 2013).

1.6.3 Bacterial immune evasion strategies

There is a plethora of mechanisms that can be employed by bacteria to evade host immune defences, and these can differ between ecological niches within the host (Figure 1.9). Mechanisms commonly utilised by bacteria at sites throughout the body include the expression of surface proteins that are recognised by immune defences as 'self' antigens, known as host mimicry (Foster, 2005) and cleavage of host proteins (Lambris et al., 2008). For bacteria within the blood, many are able to promote their survival via evasion of both the complement system and neutrophil-mediated killing (Castanheira & Kubes, 2019).

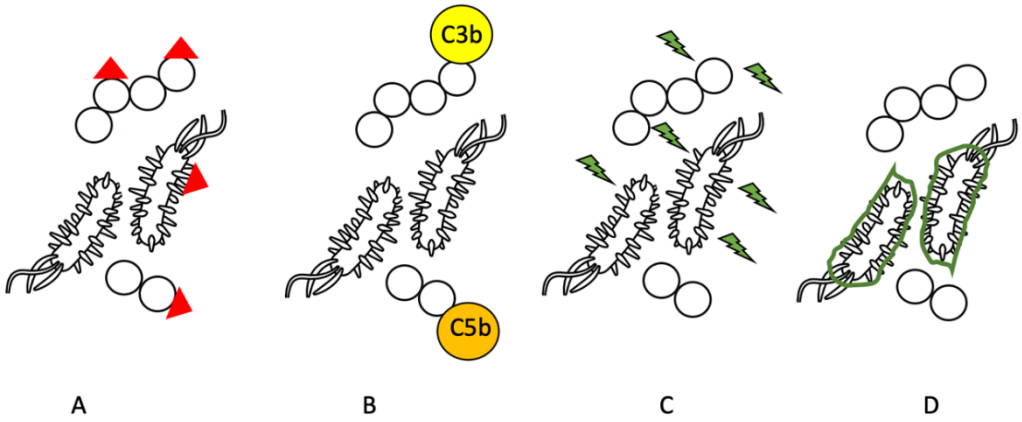


Figure 1.9 – Simplified schematic to illustrate bacterial immune evasion strategies. Some bacteria express molecules on their surface that are alike to those found on host tissues (A), whilst others may bind and present host proteins such as complement C3b (B); these two mechanisms are described as 'host mimicry'. Bacteria can secrete toxins that damage host immune components (C). Certain bacteria produce a capsule that provides protection as a thick, outer layer on the bacterial cell surface, which is difficult to penetrate and may camouflage the bacterial cell from the host immune system (D). Based on Foster (2005), Lambris et al., (2008) and Abranches et al. (2018).

1.6.3.1 Evasion of the complement system

Bacteria utilise a variety of mechanisms to evade the complement system, such as producing surface proteins or inhibitors that target complement proteins, mimicry of the complement system, or producing proteases to degrade complement proteins (Lambris et al., 2008). It is usually the complement components that function as part of a feedback loop or as a regulator that are targeted by bacteria and exploited for their advantage (Schmidt et al., 2015). For example, Factor H (FH) is a soluble negative regulator of the alternative complement pathway that protects host cells from 'self-attack'. It works in a feedback loop by recognising the increase of C3b, which opsonises bacteria for destruction by the complement system, and prevents excess generation of C3b by inhibiting C3 convertase activity (Figure 1.10) (Fallis, 2013; (Józsi, 2017).

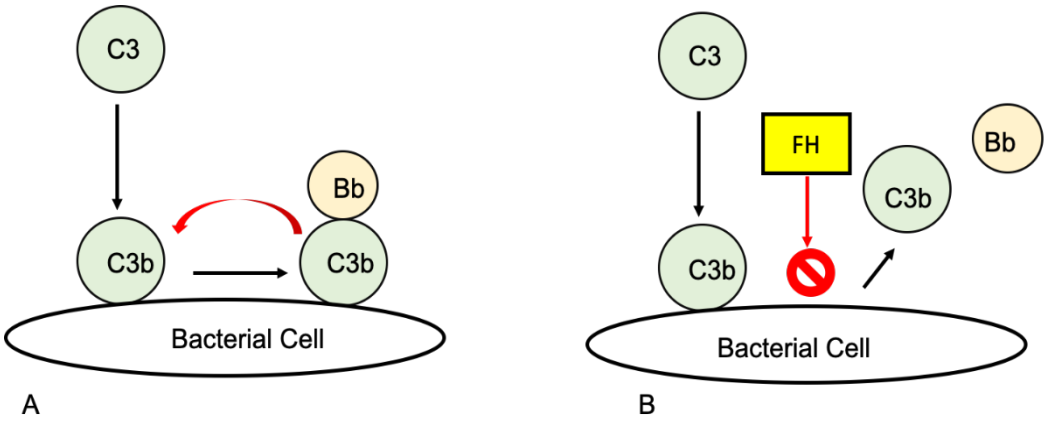


Figure 1.10 – Simplified schematic to illustrate C3b and Factor H interactions. In the alternative pathway, hydrolysed C3 and factor B leads to the subsequent formation of C3 convertase C3bBb, which converts C3 to C3b. C3b is deposited on the bacterial cell surface, opsonizing it and marking it for destruction by the innate immune system (A). Factor H (FH) is a soluble negative regulator of this pathway and recognises the increase in C3b deposition on the cell. After recognition of the C3b increase, FH prevents further C3b production by inhibiting C3 convertase activity (B). Based on Fallis (2013) and Józsi (2017).

Because FH can prevent deposition of C3b, many bacteria have evolved the ability to bind directly to FH and so protect themselves from opsonisation. This is seen with *Streptococcus pyogenes*, which expresses a Factor H-binding protein (Fba) that can bind to both FH and Factor H-like protein (FH-L). Studies have shown that Fba and FH-L work in unison to facilitate the entry of *S. pyogenes* into epithelial cells, as well as preventing surface opsonisation and subsequent phagocytosis (Pandiripally et al., 2003). There is also evidence to suggest that the fibrillar M protein found on the surface of *S. pyogenes* may bind to FH and prevent C3b deposition (Gustafsson et al., 2013). It should be noted, however, that not all microbial interactions with FH confer a survival advantage. Some microbes, such as Group *B Streptococcus* (GBS), have been shown to bind to FH via surface proteins to promote complement degradation. GBS incubated with pure FH was shown to degrade almost 90% of C3b during *in vitro* work. This is highly advantageous to bacteria, as degradation of C3b results in minimal identification and destruction by the complement cascade (Maruvada et al., 2009).

1.6.3.2 Evasion of neutrophils

Some bacteria have evolved the ability to evade neutrophil responses, usually by avoiding recognition and/or clearance by the neutrophil (Brinkmann & Zychlinsky, 2012). Some bacteria, in particular streptococci, can use peptidases to target neutrophil chemoattractants. For example, *S. pyogenes* expresses ScpA, which specifically degrades C5a and interleukin-8 (IL-8), which function to attract neutrophils to a site of infection. *S. pneumoniae* produces zinc metalloproteinase C (ZmpC), which is able to impair initial neutrophil rolling and decrease likelihood of NET formation (Hirschfeld et al., 2017). Some bacteria such as *S. sanguinis* utilise

nucleases, such as SWAN, which can degrade the DNA component of the NET, utilising Ca^{2+} and Mg^{2+} ions that have been sequestered from the host for optimum activity (Morita et al., 2014).

1.7 Utilising TraDIS to define essential genes

TraDIS, or Transposon Directed Insertion Site Sequencing is a novel, high throughput technique which allows for the generation and sequencing of a large transposon mutant library, which provides insight into gene essentiality within a bacterial genome. Transposons, or 'jumping genes' are DNA sequences which can change position within a genome, often resulting in mutations (Charbonneau et al., 2017). Transposons are inserted into the bacterial chromosome (often via plasmids) and ultimately disrupt the genome at several points. Bacterial cells that contain these transposons are cultured, genomic DNA is sheared, and PCR amplification of the transposon-containing fragments is carried out. This data is then analysed using Illumina sequencing and mapped against the relevant bacterial genome (Dembek et al., 2015).

Viable mutants will contain transposons that when inserted into a non-essential gene, will have no impact on the growth of the bacterium. However, when a transposon has inserted into an essential gene, causing disruption, this may be lethal for the bacterium. Bacterial colonies containing the transposons can be pooled together, providing a saturated transposon mutant library which permits the simultaneous sequencing of a large number of insertion sites. These mutant libraries can then be exposed to experimental conditions, such as blood, or saliva, to allow

for the relative fitness and potential essentiality of each gene to be determined (Dembek et al., 2015).

This technique has been proven successful in identifying essential genes responsible for Strangles in horses, an infectious respiratory disease caused by *Streptococcus equi* (Charbonneau et al., 2017). Using this technique, essential genes required for the survival of *S. equi* in both growth media and human saliva were established by mapping the TraDIS transposon insertion readouts against the bacterial genome (Zhu et al., 2017).

1.7.1 TraDIS and *S. gordonii*

So far, the gene essentiality of *S. gordonii* is yet to be determined using TraDIS and currently very little is known about the ability of this streptococci species to survive and replicate within the bloodstream. Based on previous studies by Charbonneau et al., 2017, it was proposed that the gene essentiality of *S. gordonii* to survive in both growth media and human blood could be determined using the TraDIS technique, following an adapted protocol obtained by the Waller group in Cambridge. The information obtained from TraDIS would be vital in determining further the role of *S. gordonii* in both bacteraemia and Infective Endocarditis.

Based on previous studies, it can be hypothesised that genes encoding for surface adhesins PadA, Hsa, SspA/SspB, CshA and CshB may be identified as essential for the survival of *S. gordonii* within the bloodstream (Haworth et al., 2017, Jakubovics et al., 2009 and McNab et al., 1996). However, additional genes would also be expected, such as metal-binding or stress response genes.

1.8 Project aims and objectives

Viridans group *Streptococcus* bacteria, such as *S. gordonii*, account for 40-60% of IE cases. This is explained by their capacity to transit within the bloodstream from the oral cavity to the cardiovascular system, where they can then bind and activate platelets to trigger the formation of thrombotic vegetations on heart valves, leading to heart failure. For *S. gordonii,* this unwanted thrombosis has been shown to be mediated by two surface protein adhesins, Hsa and PadA. As an IE pathogen, it is clear, however, that *S. gordonii* must be able to utilise additional strategies to survive within the bloodstream and associate with cardiac endothelium. This aim of this project was therefore to investigate these potential mechanisms in more detail so as to provide a better understanding of the molecular basis of such processes.

Specifically, the experimental objectives of this project were to:

· Utilise a barcoded transposon directed insertion-site sequencing (TraDIS) system to define the genes that are critical for *S. gordonii* survival within human blood.

· Investigate the role of specific *S. gordonii* surface proteins in promoting survival within human serum.

· Interrogate proteomics data to determine the capacity for specific *S. gordonii* surface proteins to interact with components within plasma and aortic endothelial cells.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

All bacterial strains used in this project are listed in Table 2.1. For *S. gordonii*, broth cultures were grown in Brain Heart Infusion broth supplemented with 0.5% (w/v) yeast extract (BHY). For maintenance on BHYN agar plates, BHY was supplemented with 0.5% (w/v) neopeptone and 15 g/L granulated agar. Where required, media were supplemented with 0.5 or 5 µg/ml erythromycin, 250 µg/ml kanamycin or 100 µg/ml spectinomycin. Broth cultures were routinely incubated for 16 h at 37°C in reduced oxygen conditions. For *E. coli,* broth cultures were grown in Luria-Bertani (LB) broth and maintained on LB agar plates. Where required, media were supplemented with 0.5 or 5 µg/ml erythromycin. Broth cultures were routinely incubated for 16 h at 37°C in aerobic conditions with agitation (220 rpm).

Table 2.1 Bacterial strains used in this study

University of Bristol (UB) number	Organism	Strain	Relevant genotype	Reference or Source
1507	*S. gordonii*	DL1 (Challis)	Wild-type, parental strain	(Pakula & Walczak, 1963)
2864	*S. gordonii*	DL1	Δ*padA*	Laboratory strain
2930	*S. gordonii*	DL1	Δ*hsa*	Laboratory strain
2773	*S. gordonii*	DL1	Δ*padA*Δ*hsa*	(Jung et al., 2015)
2724	*S. gordonii*	DL1	Δ*padA::aad9-pMSP-padA*	Haworth *et al.*, (2017)
2937	*S. gordonii*	DL1	Δ*hsa::aad9-pMSP-hsa*	Laboratory strain
2993	*E. coli*	TG1	Host strain, RepA$^+$	A. Waller, AHT
3009	*E. coli*	TG1	pGH9::IS*S1*-CA	This study
3014	*E. coli*	TG1	pGH9::IS*S1*-CT	This study
3016	*E. coli*	TG1	pGH9::IS*S1*-GA	This study

2.2 Growth curves

For growth curves, only wild-type *S.* gordonii was utilised, to give an indication of how long the bacterium takes to reach mid-exponential growth-phase. Overnight broth cultures were harvested by centrifugation (5000 *g*, 7 minutes) using a Rotina 380 benchtop centrifuge (Hettich, Massachusetts, USA), washed once with PBS and bacteria resuspended in BHY to an optical density reading at 600 nm (OD_{600}) of 1 (equivalent to approximately 10^9 CFU/ml). Suspensions were then diluted 1 in 10 into fresh, pre-warmed BHY and incubated in a candle jar at 37°C. OD_{600} values were measured every 30 minutes over the course of 8 hours using a spectrophotometer. For some studies, alongside the OD_{600} readings, every hour, viable counts were determined. At each hourly time point, an aliquot (100 µl) of bacterial suspension was transferred to a 96-well microtitre plate and serially diluted 1 in 10 into fresh BHY broth. Each dilution (3 x 20 µl) was plated onto BHYN agar, incubated for 24 hours at 37°C in a candle jar, and CFU/ml determined.

2.3 Bacterial survival in human serum

Overnight BHY broth cultures were sub-cultured into fresh BHY broth and incubated at 37ºC for 3-4 hours until an OD_{600} of approximately 0.7 was obtained, corresponding to mid-exponential phase growth. Cells were harvested (5000 *g*, 7 minutes), washed with PBS and resuspended in PBS to OD_{600} 1. Bacterial cells then were serially diluted into PBS to obtain suspensions at approximately 10^4, 10^5 and 10^6 CFU/ml. Adjusted bacterial suspensions (10 µl) were added to the wells of a 96-well plate containing 90 µl human serum (CS500-100, TCS Biosciences, Buckingham, UK) and incubated at 37°C for 0, 2 or 4 hours in the presence of 5%

CO_2, either with 80 or 150 rpm shaking. At each time point, aliquots (20 µl) of the bacteria/serum suspensions were removed, serially diluted in PBS and plated onto BHYN agar plates. The plates were incubated for 24 hours at 37°C and CFU/ml determined by viable count. During optimisation, these assays were also performed directly using bacterial cells harvested directly from overnight broth cultures.

2.4 Neutrophil studies

2.4.1 Isolation of human neutrophils

Human blood (20-50 ml) was collected into vacutainer tubes containing K_2EDTA and subsequently layered over Histopaque 1119 (Sigma, USA) and centrifuged (800 *g*, 20 minutes) at room temperature. Plasma and peripheral blood mononuclear cells (PBMCs) were removed by vacuum aspiration, with the neutrophil layer transferred into a new 50 ml falcon tube. PBS (Ca^{2+} and Mg^{2+} free) and 0.2% (v/v) human serum albumin (HSA) was added and the suspension was centrifuged again (400 *g*, 10 minutes). A Percoll gradient (Sigma, USA) of 85% to 65% was prepared in a 15 ml falcon tube, and the cell suspension was resuspended in PBS-0.2% HSA and added to the gradient. The gradient was then centrifuged (800 *g*, 20 minutes), after which the neutrophil layer was carefully removed and washed, centrifuged again (400 *g*, 10 minutes) and resuspended in 2 ml PBS. Neutrophils were diluted 1:10, counted with an improved Neubauer haemocytometer, and subsequently seeded in well plates for the experiments.

2.4.2 Preparation of bacterial biofilms for neutrophil assays

Overnight broth cultures were harvested by centrifugation (5000 *g*, 7 minutes), washed once in PBS, and resuspended in 50:50 BHY:RPMI medium (Thermo-Fisher, USA). Suspensions were then incubated at 37^{o}C for 3-4 hours until OD_{600} 0.7 was reached (mid-log phase). Suspensions were washed once with PBS, resuspended in BHY/RPMI medium to OD_{600} 1, and then 1 ml was added to wells of a 24-well plate containing 13 mm glass coverslips. The plate was incubated for 1 hour at 37°C, 5% CO_2 in humidified conditions. Wells were then washed twice with PBS to remove non-adherent bacteria, fresh BHY/RPMI was added, and the plate was incubated for a further 16 hours. Wells were then washed with PBS, fresh PBS was added, and the plate kept on ice until required.

2.4.3 Immunostaining of Neutrophil Extracellular Traps (NETs)

Bacterial biofilms were seeded with neutrophils (10^5 neutrophils per coverslip) for 2 hours, before fixing with 2% paraformaldehyde for 20 minutes. Coverslips were then washed with PBS and all cells permeabilised with permeabilization buffer (0.5% Triton-X100 in PBS) prior to washing in triplicate. Coverslips were blocked with 3% bovine serum albumin (BSA) for 30 minutes at room temperature and then incubated for 2 hours with 50 µl of primary mouse anti-human 3D9 clipped histone antibody (unpublished antibody; Max Planck Institute for Infection Biology, Berlin, Germany) at a concentration of 1.413 mg/ml. After washing in triplicate, the secondary antibody (anti-mouse Alexafluor conjugate; Life Technologies, USA) at a

dilution of 1:500 was added for 1 hour in the dark. Coverslips were washed three times, including DNA dye Hoechst 33342 (100 ng/ml; Sigma) in the first wash. After rinsing with distilled water, coverslips were mounted using Mowiol mounting medium onto microscope slides and sealed with clear nail polish for 30 minutes prior to visualisation by fluorescence microscopy.

2.4.4 Neutrophil oxidative burst assay

Bacteria required for the assay were harvested from overnight broth cultures by centrifugation (5000 *g*, 7 minutes), washed once in PBS, and resuspended in RPMI medium to OD_{600} 1. Cells were then serially diluted in RPMI to obtain a suspension of approximately $5x10^6$ CFU/ml, and added to 50% human serum for 15 minutes at 37°C, 5% CO_2 to induce opsonization prior to use. Neutrophils in 100 µl HBSS (with Ca^{2+} and Mg^{2+}) supplemented with 0.025% HSA were seeded at a density of 10^5 per well in a white 96-well plate. Horseradish peroxidase (HRP) and luminol were diluted 1:200 and 11 µl added to each well. The plate was then incubated for 15 minutes at 37°C. Phorbol myristate acetate (PMA; 5 µl, 200 nM stock) was added as a positive control, and wells containing neutrophils only were used as a negative control. Opsonized bacteria (2 µl) were then added to the remaining wells at a multiplicity of infection (MOI) of 1:20 bacterial cells to neutrophils. Chemiluminescence was recorded over 4 hours at 2.5-minute intervals using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany).

2.4.5 Ethical approval

Anonymised blood from healthy donors was collected according to the Declaration of Helsinki. The study was approved by an NHS Research Ethics Committee and was agreed to be carried out at the University of Bristol.

2.5 Vitronectin binding assay

Multimeric vitronectin (Merck) was diluted in Tris-buffered saline supplemented with 5 mM calcium chloride (TBSC) to a final concentration of 10 µg/ml and incubated with an equal volume of heparin (180 µg/ml; Sigma) or TBSC alone as control for 1 hour at 37°C with gentle agitation (50 rpm). Bacteria were harvested from overnight broth cultures (5000 *g*, 7 minutes), washed with TBSC, resuspended in TBSC to OD_{600} 1, and aliquots (50 µl) added to the wells of an Immulon 2HB 96-well plate. Following incubation at 37°C for 1-2 hours, unbound bacteria were removed by washing wells once in 200 µl TBSC, and non-specific binding sites were blocked with 3% (w/v) BSA in TBSC for 1 h at 37°C. Wells were washed with TBSC before 200 µl multimeric vitronectin ± heparin was added and incubated for 1 h at 37°C. Unbound protein was removed, and wells washed once in TBS (200 µl). Primary antibody (50 µl, mouse anti-human Vn; Santa Cruz Biotechnology, Texas, USA), diluted 1 in 1000 into Tris-buffered saline supplemented with 0.1% Tween-20 (TBST), was added to the wells and incubated for 1 h at 37°C. Wells were washed twice in TBST before adding secondary antibody (50 µl, goat anti-mouse-HRP; Dako, Denmark) diluted 1 in 2000 into TBST containing 3% (w/v) BSA and incubating for a further 1 h at 37°C. Wells were washed once in TBST and then twice

in TBS. ELISA reagent (0.102 M Na_2HPO_4, 0.049 M citric acid, 0.012% H_2O_2, 3.7 mM *O*-phenylenediamine; 50 µl) was added to wells and incubated in the dark for 10 minutes at room temperature. The colorimetric reaction was then stopped by addition of H_2SO_4 (0.65 M, 50 µl), and levels of adhesion were quantified by measuring absorbance at 490 nm using an iMark Microplate Absorbance Reader (Bio-Rad, USA).

2.6 TraDIS studies

2.6.1 Preparation of competent *E. coli* cells

An overnight LB broth culture (5 ml) of *E. coli* was sub-cultured 1:50 into fresh, warm LB broth and incubated at 37ºC, 220 rpm for 1.5-3 hours until an OD_{600} of approximately 0.5 was obtained. Cells were harvested (3000 rpm, 15 minutes, 4°C), resuspended in 1/3 volume of ice-cold, sterile $CaCl_2$ (50 mM), placed on ice for 20 minutes and harvested again (3000 rpm, 15 minutes, 4°C). Cells were resuspended in ice-cold 100 mM $CaCl_2$/15% glycerol and divided into 100 µl aliquots, which were snap frozen in liquid N_2 and stored at -80°C.

2.6.2 Transformation of *E. coli*

Frozen aliquots of competent cells were thawed on ice and mixed with 2 µl pGhost (pGH9) plasmid DNA (obtained from the Waller group). Samples were incubated on ice for 30 minutes and then heat shocked at 42°C for 90 seconds. Cells were placed on ice for 2 minutes, before 800 µl of pre-warmed LB broth was added. Suspensions

were incubated for 45-60 minutes at 37°C with shaking at 220 rpm. Samples were then serially diluted into LB broth, plated onto LB agar supplemented with 0.5 µg/ml erythromycin, and incubated at 37°C for 24-48 hours until potential transformants were visible. Successful transformants were confirmed by extracting plasmid DNA from overnight broth cultures using a QIAprep spin miniprep kit (QIAGEN, Germany) and visualising the DNA on 0.8% agarose gels.

2.6.3 Preparation of electrocompetent *S. gordonii* cells

S. gordonii is a naturally competent organism, however, in order to make the cells electrocompetent for electroporation the following protocol was carried out. An overnight BHY broth culture (10 ml) of *S. gordonii* was sub-cultured 1:100 into fresh BHY broth and incubated at 37ºC for 3-4 hours until an OD_{600} of approximately 0.4 was obtained. Cells were placed on ice for 10 minutes prior to harvesting (3000 *g*, 10 minutes, 4°C) and then washed three times in sterile double-distilled water (ddH_2O), and then three times in ice-cold wash buffer (0.3 M sucrose, 10% glycerol). Cells were resuspended in ice-cold wash buffer (0.5 ml) and 50 µl aliquots snap frozen in liquid N_2 and stored at -80°C.

2.6.4 Electroporation of competent *S. gordonii* cells

Frozen aliquots of competent cells were thawed on ice and 1 mm electroporation cuvettes were pre-chilled. pGhost (pGH9) plasmid DNA was obtained using a Monarch Plasmid MiniPrep kit following manufacturer's instructions (New England Biolabs, Hitchin, UK). 2-5 plasmid DNA (90-100µg/ml) or ddH_2O as negative control

was added to the cells, suspensions transferred to the chilled cuvettes, and electroporated at 100 Ohms, 25 µF and 2.5 kV. BHY supplemented with 0.3 M sucrose was then immediately added to the cells and the sample placed on ice for 10 minutes. Cells were incubated for 3 hours at 28°C for pHY304 or 37°C for pMSP. After incubation, samples were serially diluted into BHY broth and plated onto BHYN agar supplemented with 5 µg/ml erythromycin. Plates were then incubated at 37°C for 24-48 hours in a candle jar until potential transformants were visible.

2.6.5 Transformation of *S. gordonii*

An overnight BHY broth culture (10 ml) of *S. gordonii* was diluted 1:40 into fresh BHY broth and mixed with 1 µl competence stimulating peptide (CSP) (DVRSNKIRLWWENIFFNKK, 100 µg/ml stock; GenicBio, Taiwan), 100 µl foetal calf serum (FCS), and 2-5 µl (90-100 µg/ml) pGhost (pGH9) plasmid DNA (or ddH_2O control). Samples were then incubated in a candle jar for 5 hours at 28°C. Samples were then serially diluted into BHY broth, plated onto BHYN agar supplemented with 0.5 µg/ml erythromycin, and incubated at 28°C for 24-48 hours in a candle jar until potential transformants were visible. Successful transformants were confirmed by extracting plasmid DNA from overnight broth cultures using a QIAprep spin miniprep kit (QIAGEN, Germany) and visualising the DNA on 0.8% agarose gels.

For initial work to determine whether transformation or electroporation was most efficient, laboratory plasmids pMSP and pHY304 were utilised, as plasmids obtained from the Waller group were low in abundance and it was critical to preserve as much as possible for TraDIS experiments. However, for all TraDIS preparation experiments, the pGhost (pGH9) plasmid from the Waller group was utilised.

2.6.6 Preparation of DNA library for TraDIS

A single *S. gordonii* transformant colony was used to inoculate BHY (10 ml) supplemented with 0.5 µg/ml erythromycin (BHY-erm) and incubated overnight at 28°C in a candle jar. The culture was heat shocked at 40°C for 3 hours and then spread in 350 µl aliquots onto approximately 30 x 150 mm diameter BHYN plates supplemented with 0.5 µg/ml erythromycin. Plates were incubated at 37°C, 5% CO_2 under humidified conditions for 24 hours, and the colonies then harvested by scraping into BHY supplemented with 25% glycerol and stored as 500 µl aliquots at -80°C. An aliquot of this suspension (20 µl) was used to inoculate 20 ml BHY-erm, incubated at 37°C to OD_{600} 0.5, and then split into 5 ml aliquots. Cells from each aliquot were harvested (10,000 *g*, 5 min) and pellets stored at -20°C ready for DNA extraction.

2.6.7 DNA preparation for TraDIS

DNA was prepared for next generation sequencing based on an adapted protocol by (Jung et al., 2015) In brief, DNA was extracted from library pellets using a GenElute Bacterial Genomic DNA Kit (Sigma, USA) and quantified using a high sensitivity Quant-iT dsDNA Assay Kit (Thermo-Fisher, USA). The DNA then underwent fragmentation, α-tailing and end repair, utilising an Ultra II DNA Library Prep Kit (New England Biolabs, USA). A Y-adaptor was generated in-house using adaptor primers 1Δ/2Δ (Table 2) and ligated to the fragmented DNA, and the fragments purified using AmPure XP beads (Beckman-Coulter, USA). Restriction digest with *Sma*I was used to cleave any plasmid pGh9:IS*S1*, and the DNA purified and quantified again. Library DNA was PCR amplified using a specific IS*S1* primer

and indexing primer, as detailed in Table 2, according to the NEBNext Ultra II Q5 Master Mix (New England Biolabs, USA) protocol. DNA was cleaned again using AmPure XP beads, the average fragment size estimated by gel electrophoresis, and then quantified using a Kapa Library Quantification Kit (Kapa Biosystems, USA).

Table 2.2 Primers used in this study

Primer	Sequence
Specific IS*S1* primer	AATGATACGGCGACCACCGAGATCTACACGTTCAT TGATATATCCTCGCTG
Adaptor primer 1Δ	P-GATCGGAAGAGCACACGTCT
Adaptor primer 2Δ	ACACTCTTTCCCTACACGACGCTCTTCCGATC×T
Indexing PCR primer 1	CAAGCAGAAGACGGCATACGAGATCGGTTCGCCT TAACACTCTTTCCCTACACGACGCTCTTCCGATCT
Indexing PCR primer 2	CAAGCAGAAGACGGCATACGAGATCGGTCTAGTA CGACACTCTTTCCCTACACGACGCTCTTCCGATCT
Indexing PCR primer 4	CAAGCAGAAGACGGCATACGAGATCGGTGCTCAG GAACACTCTTTCCCTACACGACGCTCTTCCGATCT

2.7 Statistical analyses

Statistical analyses were performed using GraphPad Prism or Microsoft Excel (Microsoft Office 365 Plus). Data shown are the mean ± standard deviation of the mean (SD) and, unless otherwise stated, experiments were performed in triplicate. Comparisons between two samples were performed using the Student's 2-tailed unpaired *t* test or, for multiple samples, using an ANOVA followed by a Tukey post-hoc test. A *P* value of <0.05 was considered significant.

Chapter 3

S. gordonii survival in blood and progression of IE

3.1 Introduction

S. gordonii is a common oral streptococcal species and also identified as an etiological agent of IE. The interaction between *S. gordonii* and platelets has been recognised as an important contributor in the pathogenesis of IE (Jung et al., 2015). Two key surface proteins found on *S. gordonii*, PadA and Hsa, mediate interactions with platelets via receptors GPIIbIIIa and GPIb, respectively (Gavillet et al., 2015). This enables *S. gordonii* to promote activation and aggregation of platelets at sites of roughened cardiac endothelium, generating a septic thrombus or vegetation (Jung et al., 2015).

Binding of *S. gordonii* to ECM components exposed during tissue injury may also help to promote thrombus formation on damaged cardiac endothelium (Gavillet et al., 2015). Again, PadA and Hsa are implicated in this process. Previous studies have shown that PadA and Hsa may work in concert to mediate *S. gordonii* binding to fibronectin and vitronectin (Jung et al., 2015). As the disease progresses, host cells such as neutrophils may be activated and recruited to the site of infection. It has been suggested that activated platelets can promote the production of NETs, which further contribute to the generation of vegetations on the endocardium, in which bacterial cells are embedded (Gavillet et al., 2015). However, the capacity for *S. gordonii* to exacerbate this process during IE has not been explored.

Alongside interactions with host cells at damaged heart valves, to contribute to IE, *S. gordonii* must survive within the hostile environment of the blood stream (Singh et al., 2010). Studies have demonstrated the capacity for strains of *S. gordonii* to evade deposition of complement opsonins (Alves et al., 2019) and survive within PMNs following adhesin-mediated phagocytosis (Jung et al., 2015). However, the molecular detail of these immune evasion strategies is not well understood.

Furthermore, *S. gordonii* has been shown to (modulate gene expression upon transition from the slightly acidic resting oral pH (6-6.5) to the neutral pH of blood (7.3) (Gavillet et al., 2015). This infers that *S. gordonii* may exploit a number of different strategies to persist within the blood stream.

One recent approach that has been utilised to identify genes involved in blood survival for *S. pyogenes, S. suis* and *S. agalactiae* (Singh et al., 2010) is a high throughput next generation sequencing (NGS) technology known as Transposon Directed Insertion Site Sequencing (TraDIS). This technology allows transposon mutant libraries to be generated, which can then be utilised to identify bacterial genes that are required under a specific condition (Jung et al., 2015). Mutant libraries are prepared using plasmid pGh9::IS*S1* (Figure 3.1), which carries barcoded variants of insertion element IS*S1*. This is a transposable element that inserts randomly into the bacterial chromosome with high efficiency and coverage (Maguin et al., 1996). Transposon libraries are grown under a specific experimental condition (e.g. in human blood), and the bacteria that are recovered are then sequenced and compared to the control library/inoculum. This generates a data set that provides information on the fitness and gene essentiality for that experimental condition (Gavillet et al., 2015). If mutants carrying transposon insertions have decreased in number, this indicates that the transposon has disrupted genes that are important for survival (Alves et al., 2019).

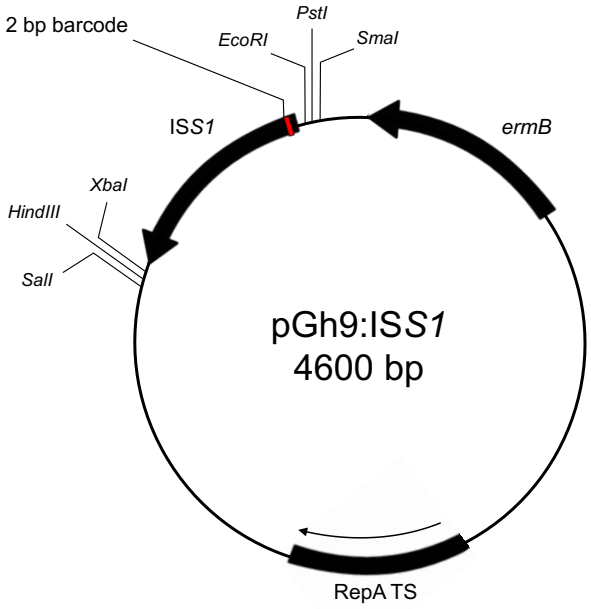


Figure 3.1 - Barcoded pGh9:IS*S1* map. pGh9::IS*S1* is a temperature-sensitive plasmid utilised for the delivery of IS*S1*. The IS*S1* transposase is flanked by an 18 bp inverted repeat and an 8 bp direct repeat. pGh9:IS*S1* carries an erythromycin resistance gene (*ermB*) used to select for successful transposition, and the Gram-positive temperature-sensitive replicase, RepA TS. Restriction enzyme sites in close proximity to IS*S1* and its 2 bp barcode are shown. Reproduced from Charbonneau et al. (2017).

The overall aim of the work presented in this chapter was to try and further understanding of the mechanisms by which *S. gordonii* can survive within the bloodstream and promote IE. Two main approaches were taken for this: 1) obtain a global overview of genes involved in blood survival utilising the TraDIS system, from which more targeted studies could then be developed, and 2) given their known association with IE, further investigate the potential functions of specific adhesins PadA and Hsa.

Specifically, the key objectives of these studies were to:

- Prepare transposon libraries in *S. gordonii* using the TraDIS system and identify the essential genome for this bacterium
- Utilise the TraDIS transposon libraries to identify genes that significantly influence *S. gordonii* survival in human blood
- Identify the potential role of *S. gordonii* adhesins PadA and Hsa in mediating *S. gordonii* survival in human serum
- Further define the molecular basis of interactions between *S. gordonii* adhesins PadA or Hsa with ECM component vitronectin
- Investigate the interactions of *S. gordonii* adhesins PadA and Hsa with neutrophils in the context of infective vegetation formation

3.2 Results

3.2.1 TraDIS studies

3.2.1.1 Survival of *S. gordonii* in serum

The first stage of these TraDIS studies was to optimise the parameters that would ultimately be used for the blood survival assays. Ethical constraints relating to blood collection within the School meant that access to healthy adult donors was limited. As such, optimisation was instead performed using human serum. Survival of *S. gordonii* in serum was determined over 6 hours. This incubation period was selected based on preliminary data within the group that indicated that viable bacteria could be detected over this time frame and was similar to other reported serum bactericidal assays (Singh et al., 2010). Three different inocula of 10^4, 10^5 and 10^6

CFU were tested, and two different incubation conditions: 37°C, 5% CO_2 with agitation at 150 rpm or 80 rpm (Trainor et al., 1999).

For the most part, levels of bacterial survival across the time period correlated with inoculum (Figure 3.2). Fluctuations in CFU were seen over the 6 hours under all conditions, but there was a general trend for viable cell numbers to remain constant or decrease up to 1 log-fold over time, the latter particularly seen with the higher inocula (Figure 3.2). The main exception to this was seen when using a 10^4 CFU inoculum of mid-exponential phase cells incubated at 150 rpm, for which a slight increase in cell viability was recorded over the 6 hours (Figure 3.2D). Levels of bacterial survival were generally comparable for the two growth phases and for the two incubation conditions.

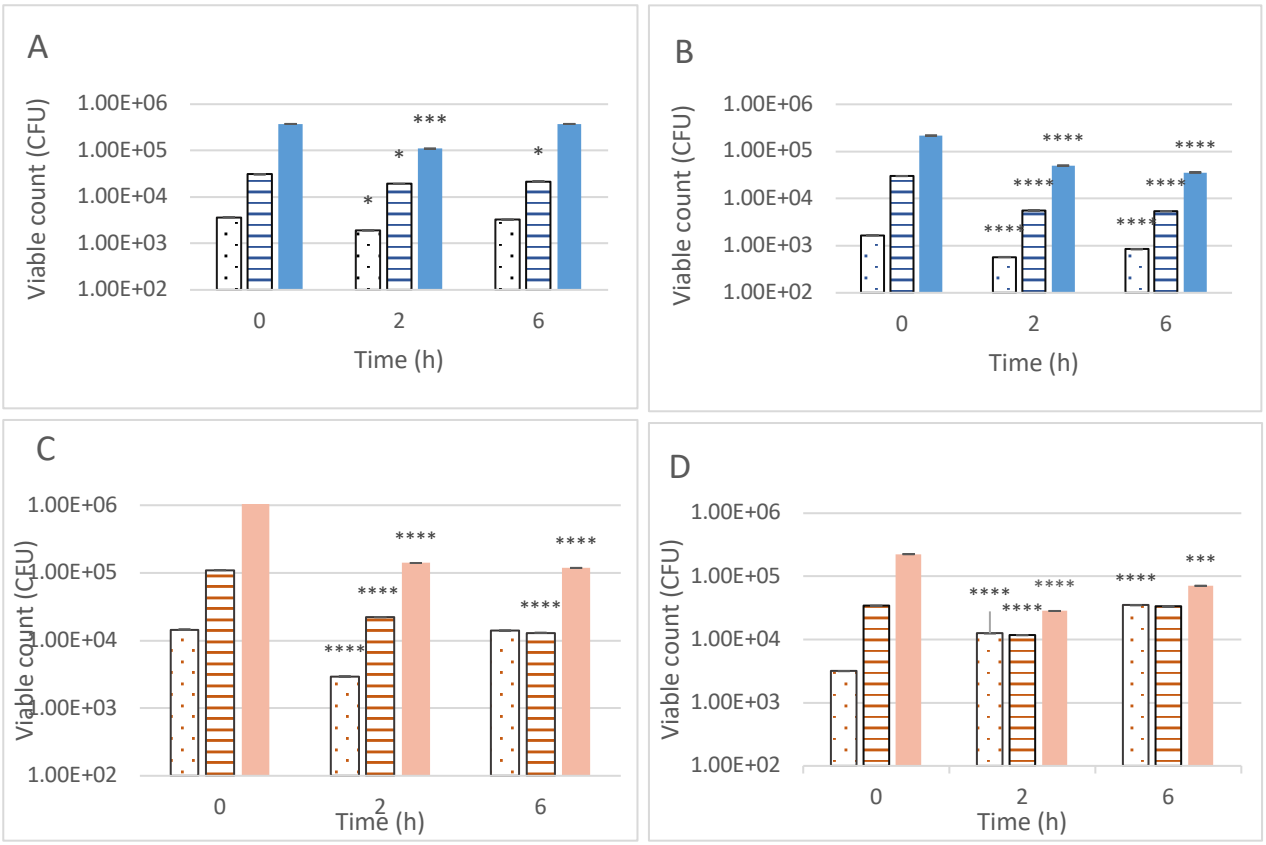


Figure 3.2 – Impact of cell density, growth phase and levels of aeration on survival of *S. gordonii* in human serum over 6 hours. Bacterial cells harvested from overnight culture (A, B) or mid-exponential phase (C, D) were adjusted to 10^4 (dots), 10^5 (stripes) or 10^6 (solid) CFU and incubated in human serum at 37°C, 5% CO_2 for 0, 2 or 6 hours at 80 rpm (A, C) or 150 rpm (B, D). At each time point, samples were serially diluted and enumerated by viable count. Data are presented as mean CFU ± SD; * P<0.05, *** P<0.001, **** P<0.0001 relative to equivalent 0-hour time point, as determined by one-way ANOVA followed by Tukey's post-hoc test; n=3 (biological repeat).

3.2.1.2 Preparation of strains for transposon library generation

Three different barcoded pGh9::IS*S1* plasmids were kindly donated by the Waller group: pGh9-GA, -CT and -CA. These were first transformed into competent *E. coli* TG1 cells, using erythromycin resistance for selection. Plasmid DNA was then extracted from successful clones, quantified and visualised by gel electrophoresis. All three plasmids generated the expected agarose gel electrophoresis profile (Figure 3.3) and were therefore ready to be used for transposon library generation in *S. gordonii.*

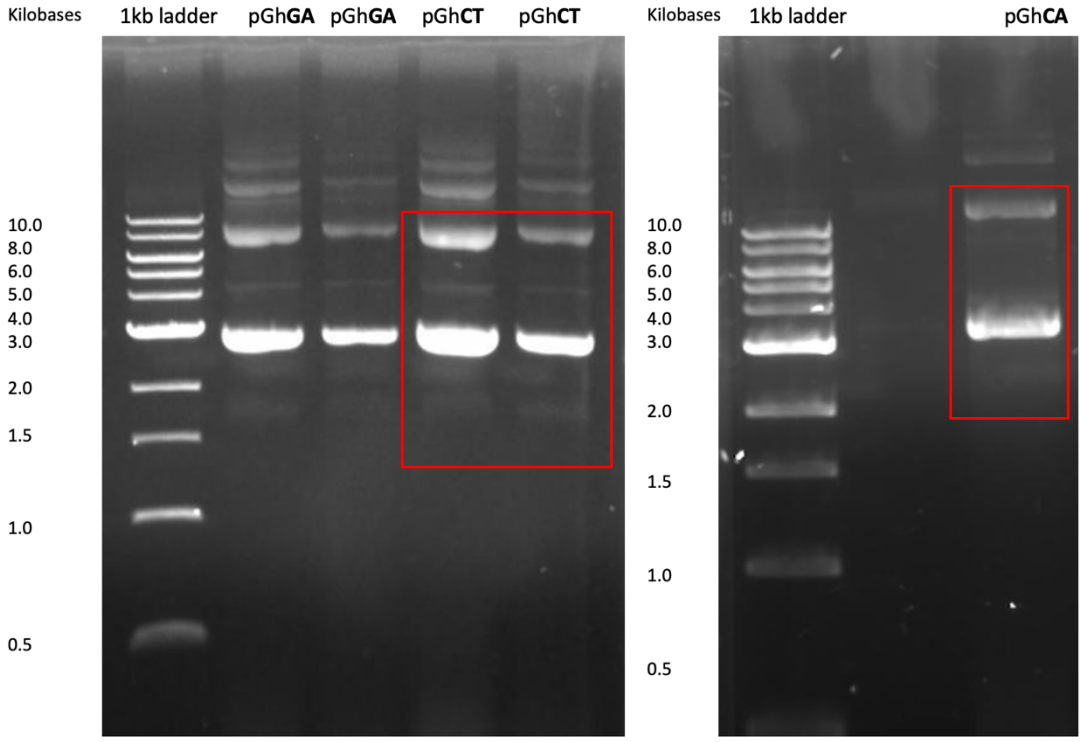


Figure 3.3 – Visualisation of barcoded pGh9::IS*S1* plasmids extracted from *E. coli*. *E. coli* TG1 was transformed with plasmids pGh9-GA, -CT and –CA and plasmid DNA subsequently extracted and visualised by agarose gel electrophoresis. All three plasmids generated the anticipated banding profile, as indicated by the red outline.

Standard transformation of *S. gordonii* is via serum-induced competence, which requires incubation periods of several hours at 37°C. However, the temperature-sensitive pGh9::IS*S1* plasmids require an incubation temperature of 28°C to maintain the plasmid in a Gram-positive host. The impact of adapting the serum-induced competence protocol for use at a lower temperature on transformation efficiency was unknown. Consequently, alongside this approach, electroporation was also tested, since this method had been used successfully for these plasmids with other streptococcal species (Jung et al., 2015).

These studies were performed prior to the pGh9::IS*S1* plasmids being received from the Waller group. As such, two alternative plasmids were selected to test these transformation protocols: pMSP7517 and pHY304. Plasmid pMSP7517 was used as it was known to transform well into *S. gordonii*, while pHY304 is another temperature-sensitive plasmid that had been used previously in *Streptococcus agalactiae*.

Compared to transformation, electroporation seemed to be of much lower efficiency (Table 3.1). When using pMSP7517, 100-fold more colonies were generated by transformation than electroporation, while no colonies were seen when using pHY304 for electroporation compared to an average of 12 colonies for transformation. This indicated that transformation by serum-induced competence would be the optimal method to use with the pGh9::IS*S1* plasmids.

	Average number of colonies (1:100 dilution)	
Plasmid	**Electroporation**	**Transformation**
pMSP7517	5	500
pHY304	0	12
ddH_2O control	0	0

Table 3.1 – Comparison of electroporation and transformation protocols in *S. gordonii*. *S. gordonii* DL1 cells were transformed with plasmids pMSP7517 or pHY304 by serum-induced competence (transformation) or electroporation. Data are presented as mean number of colonies on selective agar from a 1:100 dilution of bacterial suspension; n=2 (biological repeat).

When selecting for transformants in *S. gordonii* using erythromycin resistance, an erythromycin concentration of 5 µg/ml is used routinely, and this was used in the pilot study shown in Table 3.1. However, studies using the TraDIS system in other streptococci had performed selection with 0.5 µg/ml erythromycin. Transformation of *S. gordonii* with the three barcoded pGh9::IS*S1* plasmids was therefore performed using both concentrations for selection (Table 3.2). Both approaches generated transformants, but a higher number of colonies were recovered using selection with the lower erythromycin concentration of 0.5 µg/ml. Successful transformants were confirmed by recovery of plasmid DNA. These were then ready to be used to generate transposon libraries.

	Average number of colonies (1:100 dilution)	
Sample	**Selection at:**	
	5 µg/ml	**0.5 µg/ml**
pGh9-CT	50	370
pGh9-GA	50	500
pGh9-CA	83	500
ddH_2O (negative control)	0	0
pMSP7517 (positive control)	117	500

Table 3.2 – Transformation of *S. gordonii* with barcoded pGh9::IS*S1* plasmids. *S. gordonii* DL1 cells were transformed with plasmids pGh9-CA, -GA or –CT or pMSP7517 (positive control) by serum-induced competence and transformants recovered on BHYN agar supplemented with 0.5 µg/ml or 5 µg/ml erythromycin. Data are presented as mean number of colonies on selective agar from a 1:100 dilution of bacterial suspension; n=2 (biological repeat).

3.2.1.3 Preparation of transposon libraries

The steps undertaken to generate transposon libraries in *S. gordonii* using the three barcoded pGh9::IS*S1* plasmids are summarised in Figure 3.4 and were based on an adapted protocol (de Mattos et al., 2004). In brief, barcoded pGh9::IS*S1* transformants were heat-shocked at 42°C to induce transposition (A) and DNA extracted and quantified from the resultant transposant populations (B-D). The DNA then underwent fragmentation, α-tailing and end repair (E), followed by purification

(F). Purified DNA was then digested with *Sma*I to cleave the original pGh9::IS*S1* plasmid, followed by another round of purification and quantification. PCR utilising a specific IS*S1* primer and indexing PCR primer was then performed to amplify regions that span the 5' end of IS*S1* and the site of transposition in the *S. gordonii* genome (G). Amplicons were then purified, their size range estimated by gel electrophoresis, and quantified using qPCR (H).

The anticipated DNA yield at the endpoint (step H) should be 0.5-0.8 nM. Unfortunately, the DNA yields from these studies were in the range of 0.005-0.008 (Table 3.3) and thus 100-fold lower than anticipated. It is difficult to determine the cause of this discrepancy, but one possible point of failure may relate to the Y-adapters, which were generated in-house at the University of Bristol. To overcome this issue and complete the studies, a visit to the Waller lab had been arranged for April 2020. However, due to the unforeseen Covid-19 pandemic, laboratories underwent closure and these studies unfortunately had to be discontinued.

DNA yield (nM)		
pGh9-CT	**pGh9-GA**	**pGh9-CA**
0.00561316	0.00796206	0.00832455

Table 3.3 – Quantification of transposon library DNA for next-generation sequencing. DNA concentration (nM) was calculated at step H (Figure 3.4) of preparing transposon libraries, using an average fragment length estimation of 600 bp; n=2 (biological repeat).

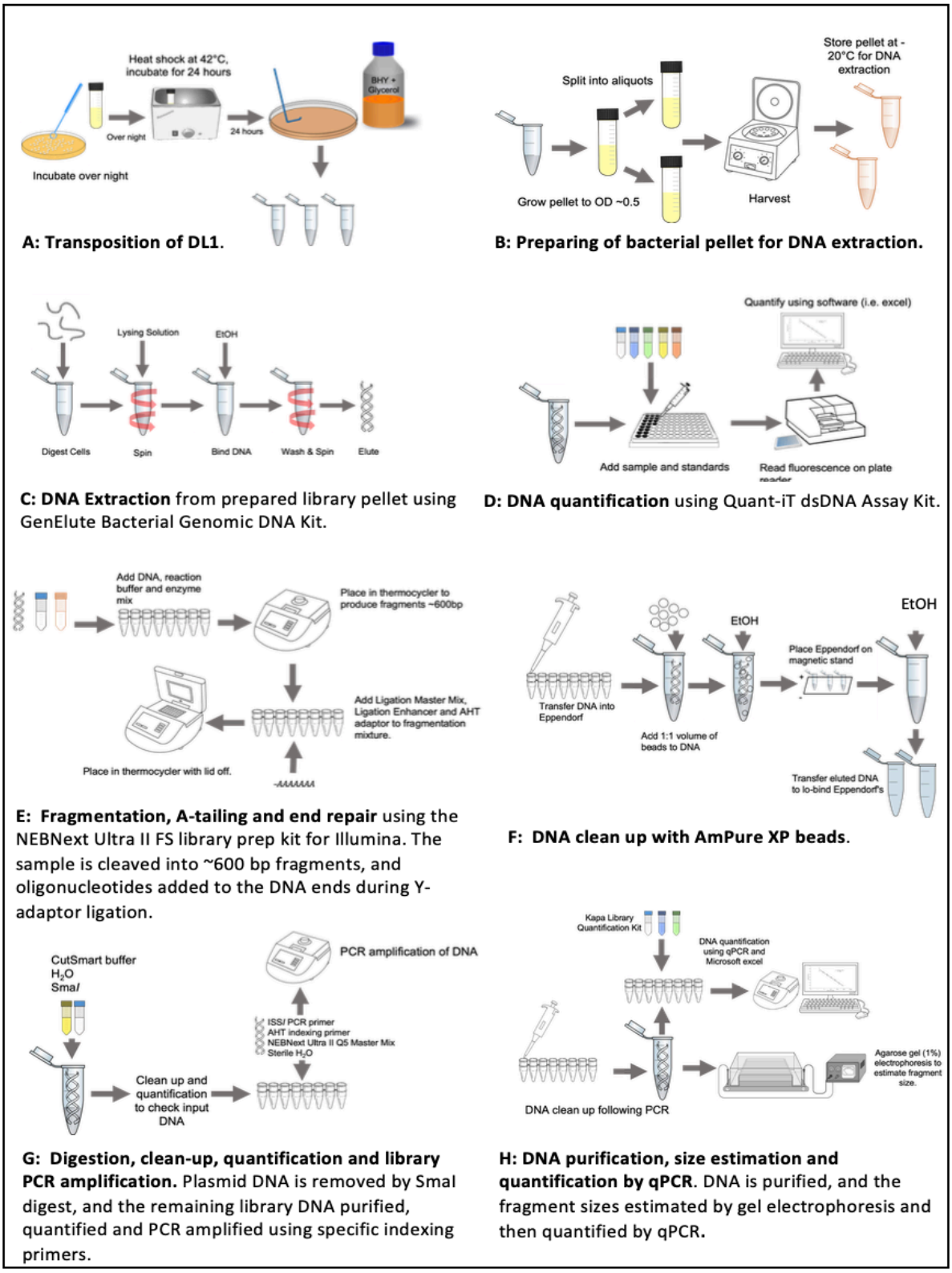


Figure 3.4 – Schematic to illustrate the steps in preparing a TraDIS transposon library and DNA for next-generation sequencing. Refer to main text for details.

3.2.2 Mechanistic basis of *S. gordonii* survival in blood

3.2.2.1 Role of *S. gordonii* adhesins in serum survival

Initial studies performed as part of the TraDIS work had indicated the capacity for *S. gordonii* DL1 to survive in human serum. Since *S. gordonii* surface proteins PadA and Hsa had previously been shown to engage with platelets within blood, it was hypothesised that these adhesins may also play a role in the survival of *S. gordonii* in serum. Based on the prior serum survival assay data, incubation conditions of 4 hours at 37 ^{o}C, 5% CO_2 with agitation at 150 rpm were selected, using a mid-exponential phase inoculum of 10^5 CFU.

To determine to roles of *S. gordonii* PadA and Hsa, survival of knockout mutants ΔPadA and ΔHsa, alongside the corresponding complemented strains +PadA and +Hsa, and a double knockout ΔPadA/Hsa strain, were compared to wild-type (Figure 3.5). Heat-inactivated serum was used as a control. Numbers of wild-type *S. gordonii* cells increased 2-fold over the 4 hours, with significantly higher levels of survival seen in heat-inactivated serum relative to untreated serum. By contrast, no increase was seen for the single or double ΔPadA/Hsa mutants. This meant that removal of either PadA or Hsa significantly reduced the number of bacteria recovered after 4 hours relative wild-type, although no additional reduction was seen upon loss of both adhesins. Levels of survival were partially restored for the two complemented mutants relative to wild-type.

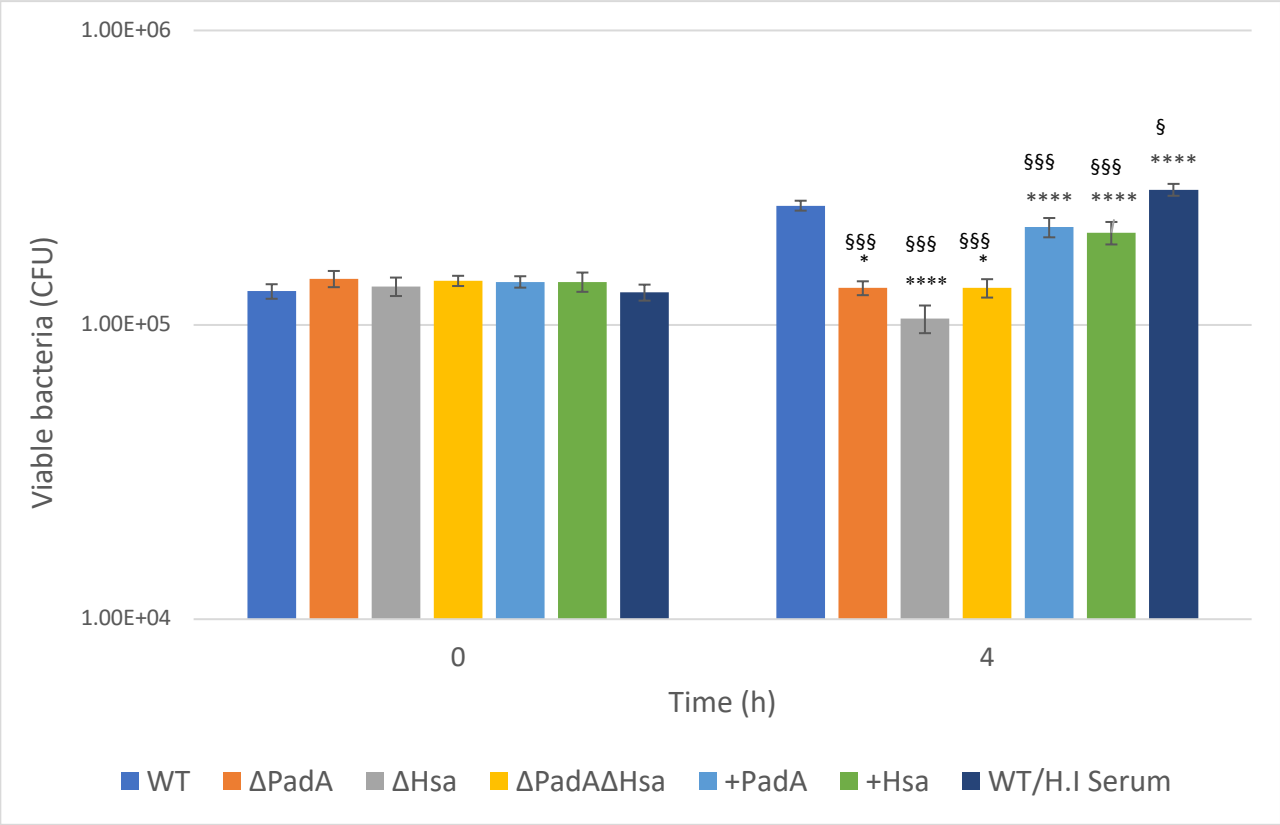


Figure 3.5 – Survival of *S. gordonii* wild-type and PadA/Hsa mutant strains in human serum over 4 hours. Bacterial cells harvested from overnight culture were adjusted to 10^5 CFU and incubated in human serum at 37 °C, 5% CO_2 for 0 or 4 hours at 150 rpm. Heat-inactivated (H.I) serum was used as a control. At both time points, samples were serially diluted and enumerated by viable count. Data are presented as mean CFU ± SD; * P<0.05 or **** P<0.0001 relative to equivalent 0-hour time point, or § P<0.05, §§§ P<0.001 relative to wild-type at 4 h, as determined by two-way ANOVA with Tukey's post-hoc test; n=4 (biological repeat).

3.2.2.2 Interactions of *S. gordonii* with vitronectin

Vitronectin is a glycoprotein found in serum, bone and the ECM. It plays an important role in coagulation and fibrinolysis following tissue injury (Gavillet et al., 2015). Vitronectin is also a regulator of the lytic pathway of the complement system, where it can inhibit C9 polymerisation and bind to the C5b-7 complex, preventing formation of the MAC (Alves et al., 2019). *S. pneumoniae* and *S. pyogenes* have been shown to utilise binding to vitronectin to promote adhesion to host cells and immune evasion (Paulsson et al., 2018; Cunha et al., 2010). Previous studies have shown that *S. gordonii* also has the capacity to bind to vitronectin, mediated by surface proteins PadA and Hsa (Haworth *et al.*, 2017). (Cunha et al., 2010)

Two regions of vitronectin have been found to be targeted by bacteria: sulphated tyrosine residues within the N-terminal domain and a C-terminal heparin-binding site (Singh et al., 2010) (Figure 3.6). Preliminary studies had demonstrated binding of *S. gordonii* to the N-terminal region of vitronectin. These studies therefore set out to determine if the C-terminal region was also targeted by *S. gordonii* and specifically PadA and/or Hsa. *S. gordonii* wild-type and ΔPadA/Hsa strains were immobilised on a microtiter plate and then incubated with multimeric vitronectin that had been preincubated with heparin or TBSC control. Wells were then washed and levels of bound vitronectin determined by ELISA. Corroborating previous data, high levels of binding to vitronectin were seen for wild-type *S. gordonii*, and these were reduced 3-fold in the presence of heparin (Figure 3.7). Likewise, vitronectin binding levels were impaired almost 3-fold for both the ΔPadA and ΔHsa mutants relative to wild-type, and these were further significantly reduced in the presence of heparin (Figure 3.7).

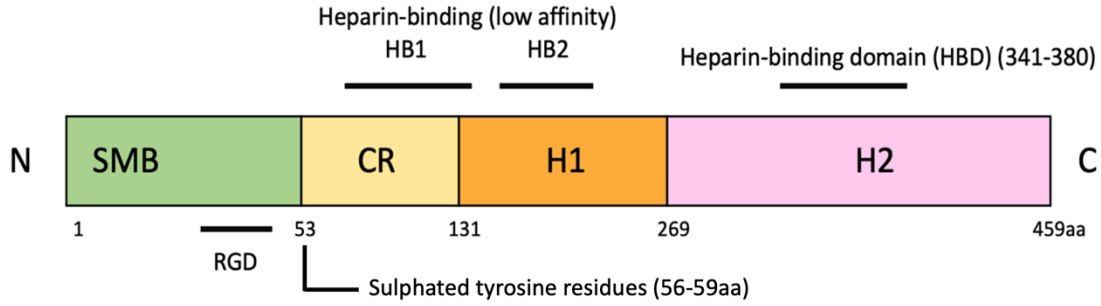


Figure 3.6 – Schematic of vitronectin indicating key domains. Domains identified at the N-terminus of vitronectin include a cell-binding motif (RGD) and a homologous region to growth-hormone intermediate somatomedin B (SMB). The connecting region (CR) connects the N-terminal with central domains, and contains acidic residues between aa 53-61, where two sulphated tyrosine residues bound by *N. meningitidis* are located at 56-59. The central and C-terminal domains contain homologous regions to plasma protein haemopexin (H1 and H2). The main heparin-binding domain is HBD, composed of highly charged residues. Low affinity heparin binding also occurs at HB1 and HB2. Of these domains, bacteria primarily target HBD. Reproduced from (Jung et al., 2015)

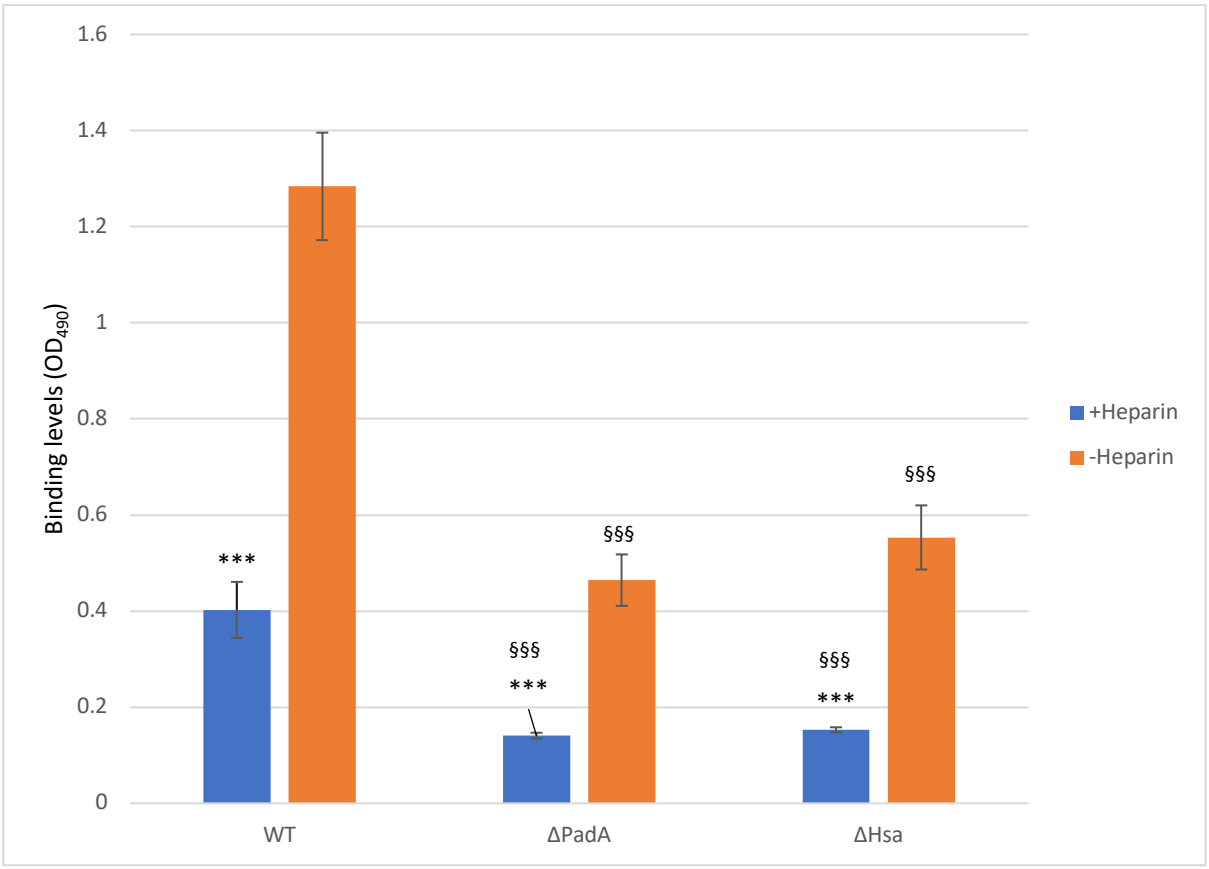


Figure 3.7 – Binding of *S. gordonii* to vitronectin in the presence of heparin. Mid-exponential phase bacterial cells were immobilised in the wells of a 96-well microtiter plate and incubated for 1 h with multimeric vitronectin ± heparin. Levels of bound vitronectin were then quantified by ELISA. Data are presented as mean absorbance ± SD; *** P<0.001 relative to no heparin control, or §§§ P<0.001 relative to wild-type, as determined by two-way ANOVA with Tukey's post-hoc test; n=3 (biological repeat).

3.2.3 *S. gordonii* interactions with neutrophils

3.2.3.1 *S. gordonii* induction of NETs

Neutrophils are amongst the first cells to arrive at a site of tissue injury and deploy a number of antimicrobial strategies, including the release of NETs to trap bacteria (Gavillet et al., 2015). However, oral *Streptococcus*, *S. mutans*, has been shown to exploit NETs to promote vegetation formation in IE via activated platelets (Alves et al., 2019). These vegetations then serve to trap and protect both bacteria and platelets. It was hypothesised that *S. gordonii* may elicit a similar effect to *S. mutans* during IE. To explore this, *S. gordonii* interactions with neutrophils were investigated. Again, given their association with IE, a potential role for *S. gordonii* adhesins PadA and Hsa was also considered.

In the first instance, to investigate whether *S. gordonii* had the capacity to induce NET formation, *S. gordonii* wild-type biofilms were grown on glass coverslips for 16 hours and then exposed to neutrophils for 2 hours. Samples were then fixed, immunostained for human histone H3 (to detect NETs) and DNA (to locate neutrophils) and visualised by fluorescence microscopy. PMA, a well-known stimulator of ROS production via activation of the NADPH oxidase complex, was selected as a positive control in these studies (Castanheira & Kubes, 2019), while unstimulated (no bacteria) neutrophils were used as a negative control. As anticipated, no NET formation was seen in the absence of neutrophil stimulation (Figure 3.8A), while abundant NETs were visible following stimulation with PMA (Figure 3.8B).

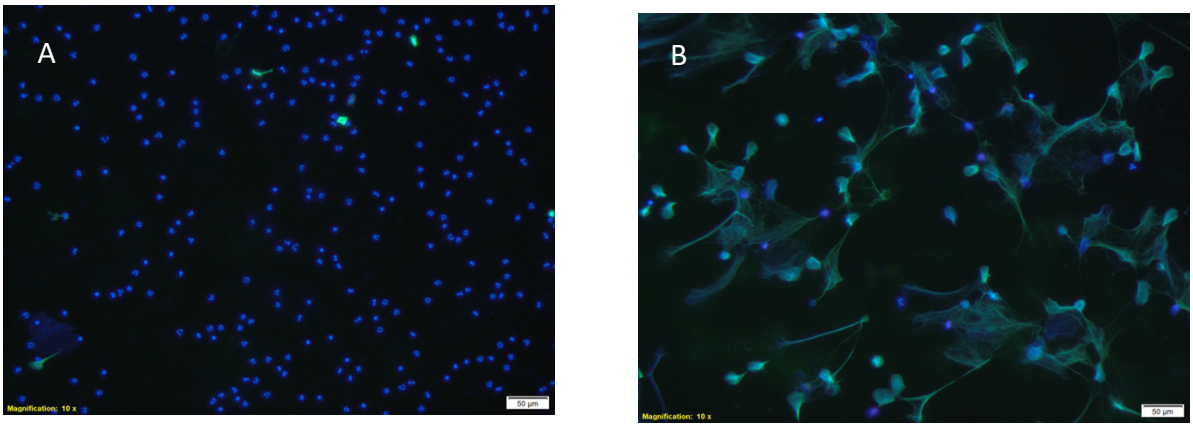


Figure 3.8 – Formation of NETs in response to PMA or no stimulus. Neutrophils were seeded onto blank coverslips in the absence (A) or presence (B) of PMA and incubated for 2 hours. Coverslips were fixed with 4% paraformaldehyde and immunolabelled for human histone H3 using an Alexa488 fluorophore (green) to label NETS and stained with DAPI for neutrophil DNA (blue). A 'cobweb' like appearance can be observed in image B with PMA, indicative of NET presence. Scale bar, 50 μm.

When neutrophils were exposed to biofilms of wild-type *S. gordonii,* induction of NET formation was clearly visible (Figure 3.9A). To explore the potential role of PadA and Hsa adhesins in this process, studies were then repeated using biofilms of the ΔPadA and ΔHsa single and double knockout mutants. Induction of NET formation was seen for each of the mutant strains, to varying extents, although in all cases levels of NET formation appeared to be lower than seen with wild-type (Figure 3.9B-D).

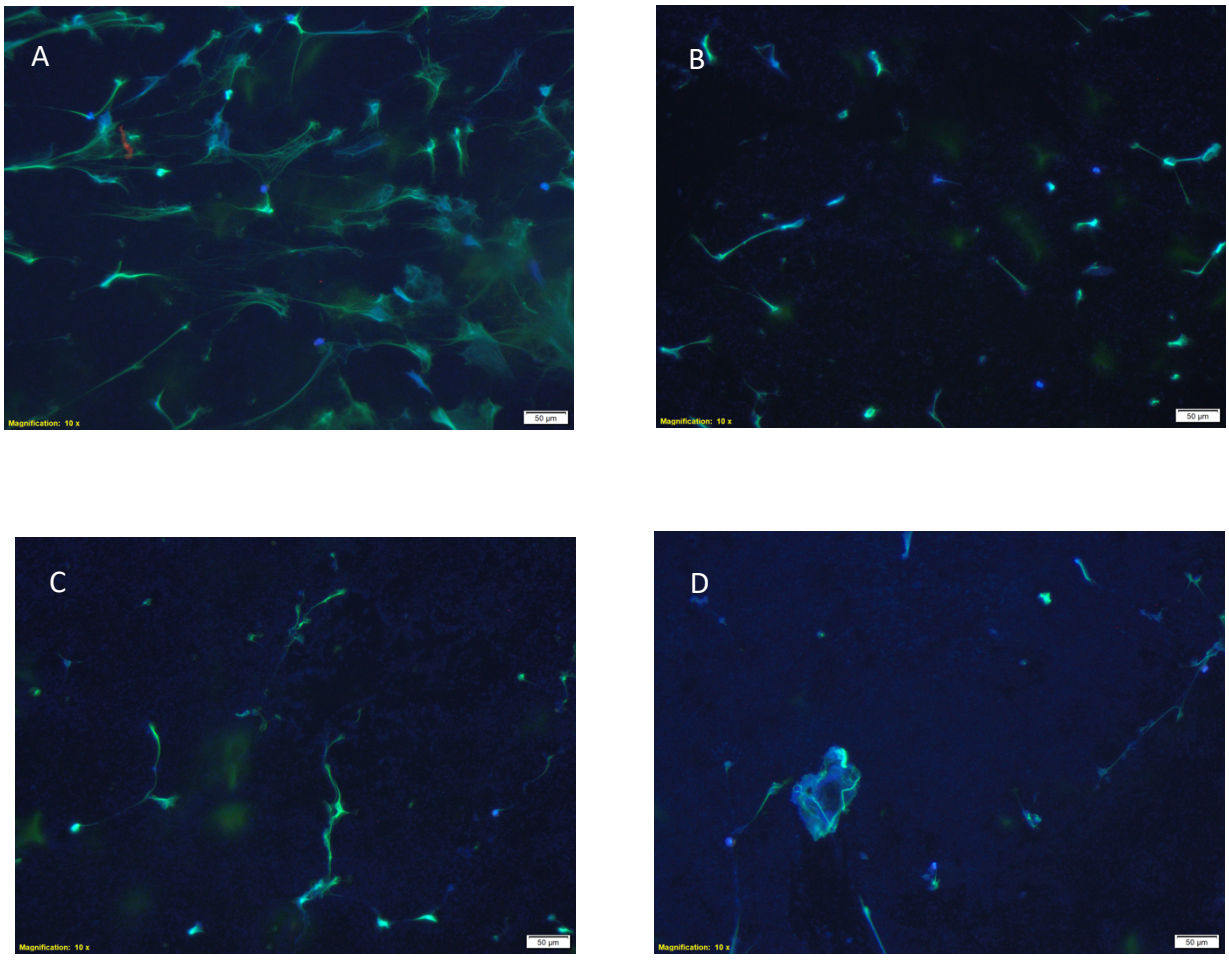


Figure 3.9 – Formation of NETs in response to *S. gordonii*. Biofilms of *S. gordonii* wild-type (A), ΔHsa (B), ΔPadA (C) or ΔPadA/Hsa (D) strains were incubated with neutrophils for 1 hour, fixed and then labelled with DAPI (neutrophil cell; blue) and Alexa488-conjugated anti-human histone H3 antibody (NETs; green). Representative images are shown. Scale bar, 50 μm.

To try and better compare levels of NET induction across the *S. gordonii* strains, the NET to background ratio was quantified using ImageJ software, whereby the image was changed to 16-bit and black and white, and the NET area manually highlighted and calculated against the background area. This semi-quantitation approach confirmed the capacity for wild-type *S. gordonii* to induce NET formation compared to the negative control (Figure 3.10). It also verified that the abundance of NETs

was significantly lower for neutrophils exposed to biofilms of each of the PadA and Hsa mutants relative to wild-type (Figure 3.10).

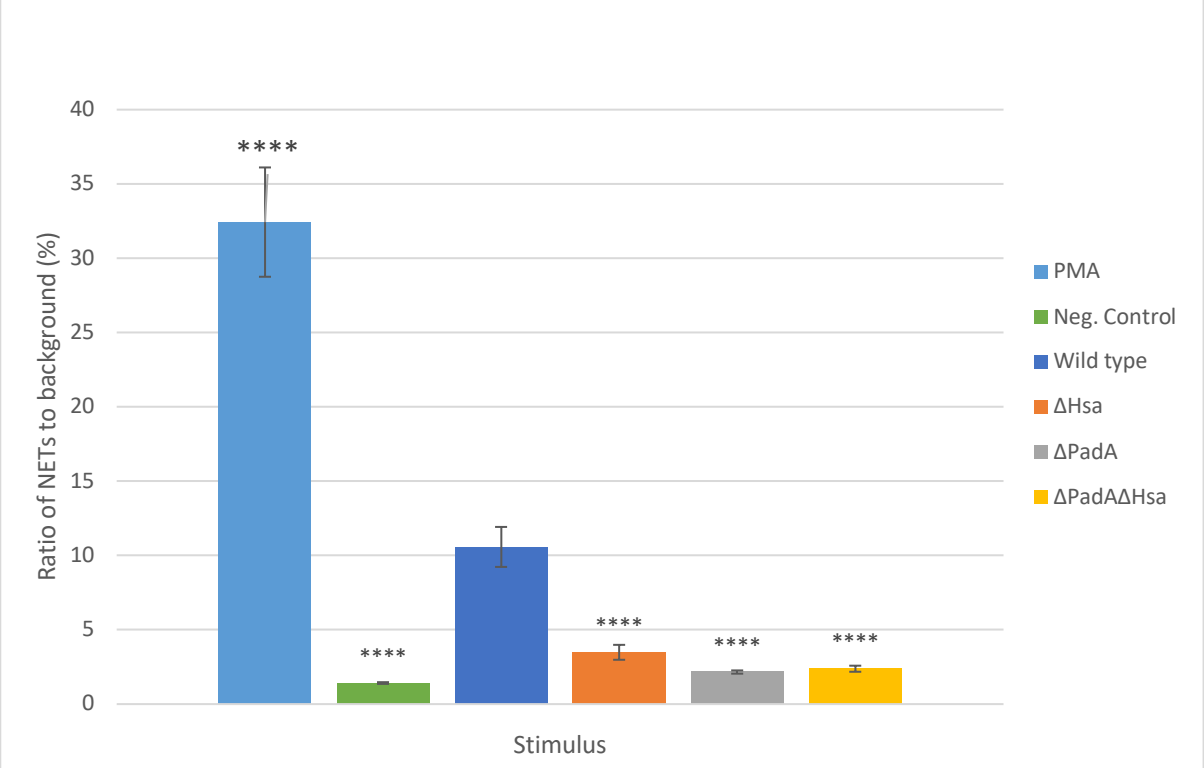


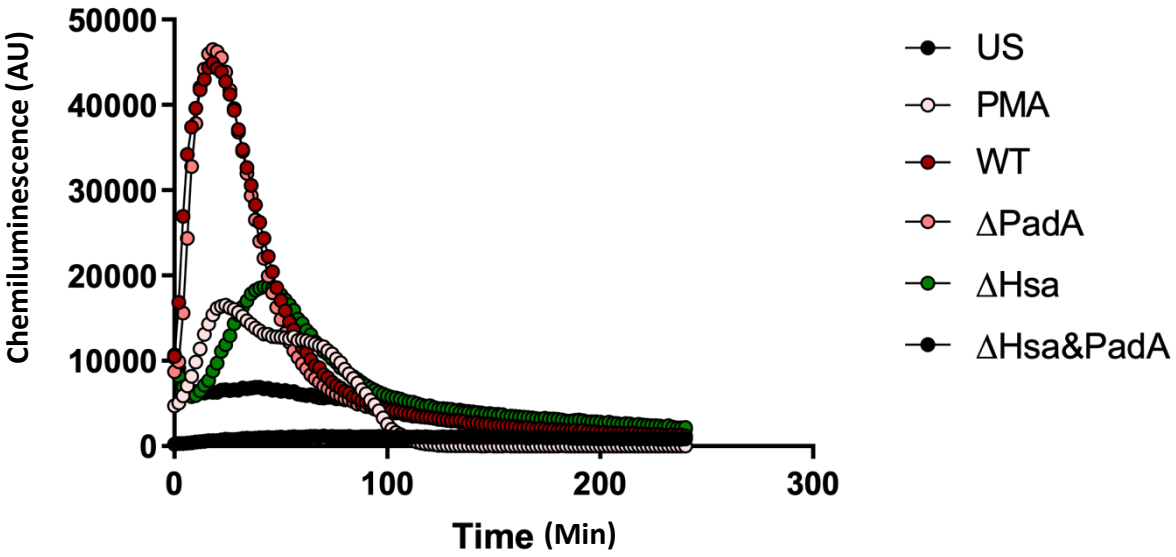
Figure 3.10 – Semi-quantitation of NET induction by *S. gordonii*. Fluorescence micrographs from the NET induction assays were uploaded to ImageJ software and converted to 16-bit and black and white. NET area was then manually selected compared to background. Data are presented as the ratio of NETs to background (12 images per sample); **** P <0.0001 relative to wild-type, as determined by one-way ANOVA with Tukey post-hoc test. n=12 (technical repeat from pooled data, collected from 3 biological repeats).

3.2.3.2 *S. gordonii* induction of neutrophil oxidative burst

To investigate the capacity for *S. gordonii* to induce the neutrophil oxidative burst, a chemiluminescence-based assay for detection of reactive oxygen species (ROS) was utilised. Bacteria were opsonised with human serum and then incubated with neutrophils to give a multiplicity of infection (MOI) of 1:20. Horseradish peroxidase and luminol were added, and chemiluminescence then recorded over 4 hours. PMA stimulation of neutrophils was used as a positive control, and unstimulated neutrophils as a negative control. The results from this assay generated chemiluminescence curves (Figure 3.11A), which were then transposed to a bar graph using area under curve (AUC) analysis (Figure 3.11B).

S. gordonii wild-type induced high levels of ROS production by neutrophils, which was higher than levels induced by PMA (Figure 3.11B). This was unexpected as PMA is a potent agonist of ROS production (Khan et al., 2014)The ΔPadA strain induced comparable levels of ROS to that of wild-type. By contrast, levels of ROS generated by neutrophils exposed to the ΔHsa strain were approximately 50% lower than for wild-type, and were reduced by approximately 75% for the ΔPadA/Hsa double knockout strain (Figure 3.11B).

A



B

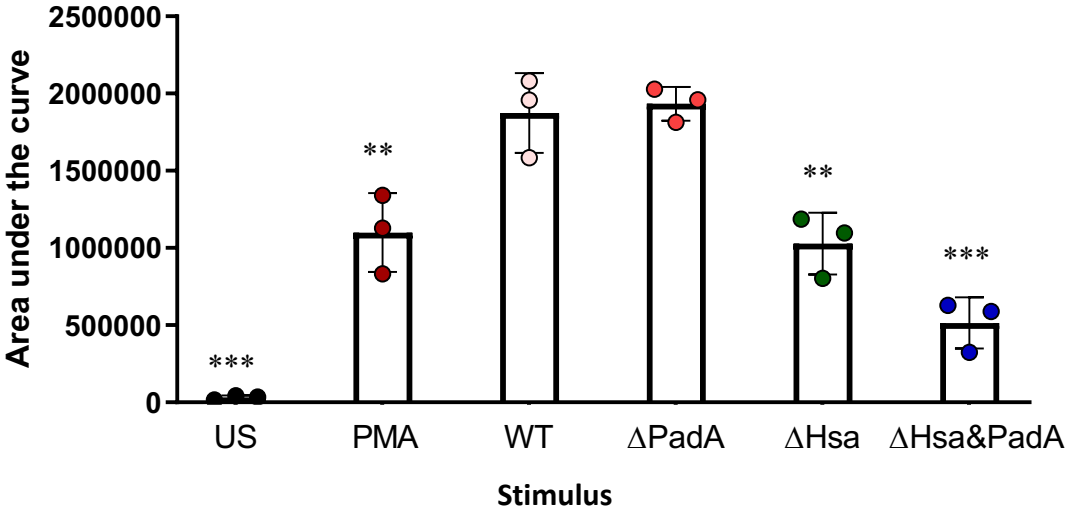


Figure 3.11 - *S. gordonii* induction of neutrophil oxidative burst. Bacterial cells were harvested from overnight cultures, opsonised with human serum, and incubated in a 96-well plate with neutrophils (MOI 1:20), horseradish peroxidase and luminol. Chemiluminescence was recorded over 4 hours. PMA stimulation of neutrophils and unstimulated neutrophils were used as positive and negative controls, respectively. Panel A shows the chemiluminescence curve and Panel B the area under curve analysis. For Panel B, data are presented as mean CFU ± SD; ** P<0.01 and *** P<0.001 relative to wild-type, as determined by two-way ANOVA with Tukey's post hoc-test; n=3 (biological repeat).

3.3 Discussion

3.3.1 TraDIS studies

The aim of these studies was to prepare a *S. gordonii* transposon library that could be exploited to gain a global overview of the genes that contribute to the survival of *S. gordonii* within human blood. Transposants using three different barcoded pGh9::IS*S1* plasmids were successfully generated, but final yields of PCR amplicons covering the sites of transposition were significantly lower than required for next generation sequencing. The reason behind this remains unclear. However, based on levels of DNA obtained at the different stages, it seems likely that either the in-house preparation of the Y-adaptor or the final PCR amplification step using the IS*S1* specific and indexing primers was the point of failure. A visit to the Waller group had been planned to troubleshoot and complete characterisation of the *S. gordonii* transposon libraries, before using them in studies involving human blood. Unfortunately, due to the circumstances imposed by the COVID-19 pandemic, this area of the investigation had to be terminated.

3.3.2 *S. gordonii* survival in serum

Human serum is obtained from blood plasma, after fibrinogen and other clotting factors have been removed. It contains a range of proteins, lipoproteins and nutrients, including a range of antimicrobial components. Key proteins found in serum that can mediate bactericidal effects include those of the complement system, so serum is often a good choice for studies relating to the complement cascade (Mathew & Bhimji, 2018). In this study, a simple serum survival assay

provided evidence that wild-type *S. gordonii* was able to survive within human serum over a 6-hour period. Some reduction in the viable population was seen, indicative of bacterial killing, but this was minimal. Frozen serum stocks were used for this work and so it might be anticipated that higher levels of killing may have been observed in fresh serum, and certainly if using whole blood, in which the cellular components of the immune system would also be available. Nonetheless, bactericidal activity of serum has been shown to be maintained for up to three freeze-thaw cycles (O'Shaughnessy et al., 2012)In general, the high level of serum survival recorded here supports the concept of *S. gordonii* as an opportunistic pathogen that is able to persist within the blood stream and so promote systemic diseases such as IE.

Heat-inactivation of serum is a technique used to investigate the role of serum proteins, namely the complement system, in mediating bactericidal effects. Higher levels of survival were seen for wild-type *S. gordonii* when incubated in heat-inactivated serum compared to untreated serum, but the difference was relatively slight. This implies that *S. gordonii* may be adept at evading complement-mediated killing. In support of this, Alves et al. (2019) recently found that strains of *S. gordonii* isolated from systemic infections could evade deposition of complement proteins C3b/iC3b and so opsonophagocytosis by PMNs (Jung et al., 2015).

Loss of either PadA or Hsa from the surface of *S. gordonii* reduced levels of serum survival relative to wild-type, while complementation increased survival. For the latter, survival was not fully restored to wild-type levels. This has been seen before with other phenotypes using these mutants (Haworth et al., 2017), and may reflect differences in adhesin expression levels when driven from the native promoter compared to the nisin-inducible promoter of the pMSP vector. Nonetheless, taken

together, these data imply that both PadA and Hsa contribute to the capacity for *S. gordonii* to persist within serum. Since no additional reduction was seen in serum survival for the ΔPadA/Hsa double mutant compared to single knockout mutants, this suggests that PadA and Hsa may exhibit cooperative functions. Again, this has been proposed for PadA and Hsa in mediating biofilm formation and binding to fibronectin or vitronectin (Haworth et al., 2017), and correlates with the vitronectin data generated here. In these studies, PadA and Hsa were both shown to bind the heparin-binding site within the C-terminus of vitronectin. However, blocking with heparin did not ablate binding, indicating that these adhesins could also target an additional site(s). This correlates with previous, unpublished data indicating that a monoclonal antibody against the N-terminal region of vitronectin could also impair attachment of the ΔPadA and ΔHsa mutants. Thus, it is possible that both PadA and Hsa must engage vitronectin simultaneously for it to mediate its protective effects. As has been reported for other bacteria (Gavillet et al., 2015), this may then enable vitronectin to protect *S. gordonii* from complement-mediated killing, providing an additional immune evasion strategy that can be exploited by this bacterium. To further explore the cooperative function of PadA and Hsa in mediating interactions with vitronectin, binding of the ΔPadA/Hsa double mutant complemented with individual Hsa or PadA proteins could be tested. If concerted action by both adhesins is required, these mutants should behave alike to the individual knockout strains.

Alongside a potential role in immune evasion, the capacity for *S. gordonii* PadA and Hsa to bind vitronectin could also enhance the capacity for *S. gordonii* to promote formation of infective vegetations during IE. Vitronectin is highly expressed within cardiac tissue and therefore likely to be exposed upon endothelial damage (Singh

et al., 2010). Attachment of *S. gordonii* to this exposed vitronectin could then facilitate initiation of thrombosis at the site of injury.

3.3.3 Interactions of *S. gordonii* with neutrophils

In response to bacteria, neutrophils deploy a variety of antimicrobial strategies, including the expulsion of NETs and production of ROS. NETs appear as 'cobweb' structures, which are primarily composed of extracellular DNA, chromatin, elastase, proteinases and histones (Castanheira & Kubes, 2019). The observation of NETs within human infective vegetations has led to the proposal that NETosis in response to bacteria and platelet activation may contribute to thrombosis during IE (Jung et al., 2015).

Strong induction of NETosis and neutrophil activation (as measured via ROS production) was seen for wild-type *S. gordonii*, even higher than that of the PMA control for ROS production. Thus neutrophil recruitment to sites of cardiac endothelial damage could exacerbate the capacity for *S. gordonii* to promote vegetation development. Furthermore, both PadA and Hsa contributed to these effects, albeit in seemingly different ways. For NETosis, loss of either PadA or Hsa significantly impaired NET formation relative to wild-type, again implying cooperative function between the two adhesins in this process. None of the mutants tested were ablated in NET induction, however, indicating that additional surface determinants of *S. gordonii* can induce NETosis. While visualisation of NETs was compelling, it is not the optimal method for quantification. For future studies, flow-cytometry using antibodies against key components of the NET structure, such as histones or granular enzymes, could be utilised (Gavillet et al., 2015). Additionally,

to further verify the role of PadA and Hsa in NETosis induction, the set of complemented mutants could be tested, alongside knockout mutants of additional surface determinants.

In contrast to NETosis, for ROS production, the PadA knockout mutant was comparable to wild-type, while a significant reduction was seen for the ΔHsa strain and this was further reduced for the ΔPadA/Hsa double knockout mutant. This infers potential redundancy of function between these two adhesins for neutrophil activation (i.e. both adhesins can independently induce neutrophil activation), but that Hsa has the dominant effect. As such, the presence of Hsa masks the activity of PadA, and this is only seen once Hsa has been deleted. (Haworth et al., 2017) To explore this further, future work could explore ROS production using the ΔPadA/Hsa double mutant complemented with individual Hsa or PadA proteins. It would be anticipated that restoration of either PadA or Hsa expression could increase levels of ROS production by neutrophils but to different levels. Again, even with the ΔPadA/Hsa double mutant, significant ROS production was seen. This indicates that additional surface determinants of *S. gordonii* can also induce neutrophil activation.

Taken together, these data indicate that upon engagement by neutrophils, *S. gordonii* is a potent activator of NETosis and neutrophil activation. Moreover, adhesins PadA and Hsa play an important but not exclusive role in mediating these effects. Activated platelets release P-selectin and type 1 β-defensins that assist neutrophil activation. Since *S. gordonii* also has capacity to activate platelets, this raises the possibility that combined challenge of neutrophils with bacteria and platelets will exacerbate NET formation. Such a mechanism has been demonstrated for *S. mutans*, another IE pathogen (Jung et al., 2015). To test this hypothesis,

studies could be repeated in which neutrophils are challenged with combinations of bacteria and platelets.

3.4 Summary

The overall aim of the work presented in this chapter was to try and further understanding of the mechanisms by which *S. gordonii* can survive within the bloodstream and promote IE. The key findings were as follows:

- *S. gordonii* DL1 possesses the ability to survive within human serum for at least 6 hours, supporting its capacity to promote systemic disease
- Adhesins PadA and Hsa contribute to *S. gordonii* serum survival
- There is evidence to suggest that *S. gordonii* can evade complement-mediated killing
- One mechanism by which this might be achieved is via cooperative binding of PadA and Hsa to vitronectin, with both adhesins able to bind the N-(sulphated tyrosine residues) and C-terminal (heparin-binding) regions of vitronectin
- *S. gordonii* can induce NETosis and ROS production (activation) when exposed to human neutrophils
- Adhesins PadA and Hsa contribute to both of these the processes
- Induction of NETosis, neutrophil activation and attachment to vitronectin are all mechanisms by which *S. gordonii* may promote the formation of infective vegetations during IE

Chapter 4

Proteomic analysis of *S. gordonii* interactions with plasma proteins and cardiac endothelium

4.1 Introduction

S. gordonii surface proteins Hsa and PadA have been extensively studied with regards to their interaction with platelets in the context of IE pathogenesis. Building on this work, preliminary studies performed within this group implied that Hsa may facilitate attachment of *S. gordonii* to human coronary artery endothelial cells (HCAEC), a function that could promote infective vegetation formation. However, the potential binding target was unknown. It was also hypothesised that Hsa and PadA may contribute to *S. gordonii* survival within the blood stream, which has now been supported by this study and the data presented in Chapter 3. To obtain a broader perspective of how Hsa and PadA may facilitate interactions of *S. gordonii* with blood components and endothelial cells, purified, native forms of these two adhesins bearing a C-terminal 6x histidine (His_6) tag were used in pull-down assays with HCAEC lysates and platelet-rich and platelet-poor plasma (PRP; PPP). The captured proteins were then analysed by nanoscale liquid chromatography with tandem mass spectrometry (nanoLC MS/MS). These experiments were performed by a former MSc student, Punkita Lohiya, but the proteomic data had not been fully analysed.

The way in which purified PadA and Hsa proteins were obtained was by engineering *S. gordonii* strains in which the LPxTG cell-wall anchor of PadA or Hsa had been replaced by a His_6 tag. This meant that upon expression, these adhesins were secreted into the culture medium, from which they could then be purified using metal affinity-based chromatography. This approach had the additional advantage that the adhesins would carry any native posttranslational modifications, which could contribute to function. This was particularly pertinent for Hsa, the gene for which is

located within an operon with a number of downstream glycosyltransferase genes that are known to act on Hsa (Figure 4.1). To further explore the role of glycosylation in Hsa function, two *S. gordonii* strains were generated: one in which the entire operon was expressed, and one in which a transcriptional terminator was placed upstream of the *gly* gene. It was anticipated that the former would express native Hsa, designated Hsa_6His(+), while the latter would express a non-glycosylated form, designated Hsa_6His(-). Both forms were used in the pull-down assays with HCAEC lysates, PRP and PPP.

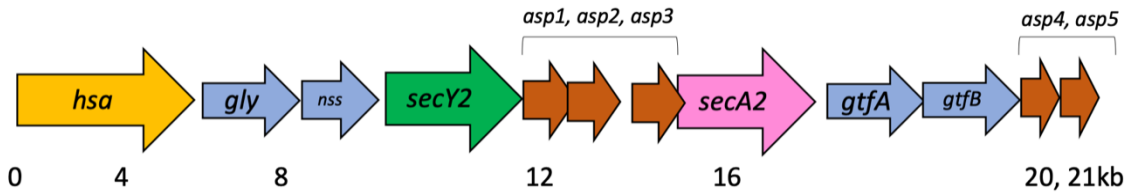


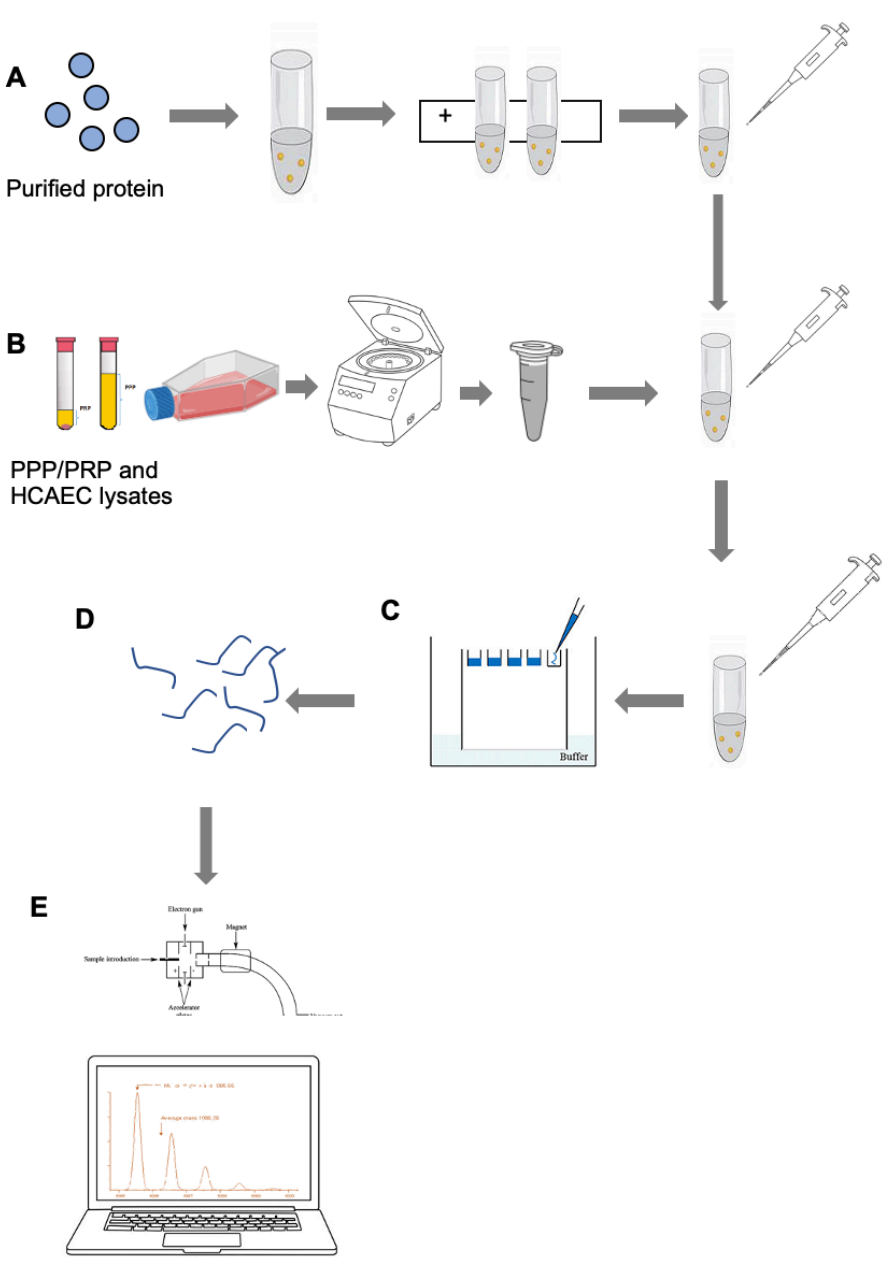
Figure 4.1 – The *secA2-secY2* locus showing genes encoding Hsa, core proteins and glycosyltransferases.

The *secA2-secY2* locus carries genes that encode Hsa, alongside accessory secretion proteins (*asp1-5*) (brown arrows) utilised for protein export and glycosyltransferases (*gtfA, gtfB, gly* and *nss*) (blue arrows), which are required for the glycosylation of Hsa. Reproduced from Bandara et al. (2016).

Eluted protein-ligand complexes from the pull-down assays had been run on a 10% SDS-PAGE gel, excised and delivered to the University of Bristol Proteomics Facility. Trypsinisation was performed to generate a collection of peptides from each sample, which were then analysed by nanoLC MS/MS. With this technique, peptides are separated by reversed-phase nano liquid chromatography, where the protein sample is in liquid phase and separated within a column. This eluent is then

continuously analysed by a mass spectrometer, which converts molecules within the eluent into a charged state. These molecules can then be analysed based on their molecular and structural characteristics; for example, the glycans within glycoproteins can be determined based on their ion content (Pitt, 2009; Rigbolt & Blagoev, 2010).

The data were filtered using a 1% false discovery rate (FDR) cut off and were compared for each His-tagged protein with the control sample using the 'area' measurement, which corresponds to relative levels of a given protein in each sample. The pull-down assays performed by Punkita Lohiya are summarised in Figure 4.2.



A – Magnetic Dynabeads coated with HsaHis$_6$(+), HsaHis$_6$(-) or PadAHis$_6$. Magnetic His-tag Isolation Dynabeads were incubated with purified proteins and washed to remove excess protein.

B – Pull-down assay. Coated beads were incubated with PPP/PRP or HCAEC lysates and unbound proteins removed by washing. Bound proteins were then eluted.

C, D – Collection of bound proteins and trypsinisation. Eluted samples were visualised by SDS-PAGE with Coomassie Blue staining and excised from the gel (C). Proteins were then de-stained and subjected to in-gel tryptic digestion (D).

E – NanoLC MS/MS analysis. Peptides were fractionated using mass-spectrometry, reduced and ionised by nano-electrospray ionization, and filtered. Raw data files were generated for subsequent analysis.

Figure 4.2 – Summary of pull-down assays using purified Hsa and PadA with PRP, PPP or HCAEC lysates to generate raw data for proteomic analysis

As the data arising from these former pull-down assays had not been fully analysed, the overall aim of the work presented in this chapter was to perform an in-depth analysis of these proteomics data to identify potential proteins within PRP, PPP and on HCAEC that may be engaged by *S. gordonii* adhesins PadA and Hsa.

Specifically, the key objectives of these studies were to:

- Use UniProt to identify potential proteins of interest on HCAEC or within PRP/PPP, based on cellular location or function, that may be bound by Hsa or PadA
- Perform Ingenuity Pathway Analysis (IPA) to identify canonical pathways involved in Hsa or PadA interactions with proteins of interest on HCAEC or within PRP/PPP
- Perform IPA to explore bio functions and their predicated activation state for proteins of interest on HCAEC or within PRP/PPP that interact with Hsa or PadA

4.2 Results

4.2.1 Shortlisting of proteins identified in PRP and PPP

Full lists of proteins bound by PadA or Hsa were initially exported to Microsoft Excel files. For PRP, 300 proteins were initially identified with Hsa_6His(+), 306 with Hsa_6His(-) and 292 with $PadA_{6His}$. For PPP, 276 proteins were identified with Hsa_6His(+), 268 with Hsa_6His(-) and 247 with $PadA_{6His}$. Lists were then further refined based on several parameters. The first parameter was the number of 'sample hits' (PSMs), which indicates the number of times a sample was detected; the higher the value, the less likely the data are to be anomalous. A cut-off PSM value of 15 was used, below which results were discounted. Alongside this, each dataset was uploaded to UniProt, a website that provides a free, accessible resource of protein functional information based on uploaded proteomics datasets. Two parameters were applied based on the Uniprot analysis, namely 'annotations'

and ‘status’. ‘Annotations’ refers to the number of times a given protein has been identified in previous users’ datasets and is indicated by a rating out of 5. For example, a 1/5 rating indicates very few to no datasets contained this protein, which may indicate contamination or an anomalous result. In contrast, a rating of 5/5 indicates that several previous datasets have contained this protein, suggesting that it has been a protein of interest in previous research and samples. The cut off for annotations was set at a 3/5 rating, a middle ground between recognised proteins and those that may not have been identified in previous studies. ‘Status’ indicates whether a protein has been reviewed or unreviewed. Reviewed refers to a sample that has been identified by both another user and the software, indicating that this sample has been identified several times in previous datasets. Unreviewed refers to a sample that has only been identified by the software, and not manually identified by another user. In order to simplify the datasets for this study, unreviewed proteins were discounted, many of which did not have protein names or descriptions. Shortlisting based on PSM value, annotations and status for $Hsa_6His(+)$, $Hsa_6His(-)$ and $PadA_{6His}$ resulted in 258, 258 and 250 proteins, respectively, for PRP, and in 222, 229 and 209 proteins, respectively, for PPP.

A final parameter used for shortlisting based on the proteomics datasets was the logarithmic fold change (LogFC) value, which indicates protein binding intensity based on the control. For example, if LogFC = 0, the ratio of sample to control is one-to-one; however, if the sample is expressed twice as much as the control, the logFC is denoted as = 1. Based on this, samples with a LogFC of below 1 were excluded. This shortlisting ultimately left 41 proteins for $Hsa_6His(+)$, 40 with $Hsa_6His(-)$ and 44 with $PadA_{6His}$ with PRP, and 51 proteins for $Hsa_6His(+)$, 51 with $Hsa_6His(-)$ and 39 with $PadA_{6His}$ with PPP.

Once the completed sets of shortlisted proteins had been generated, these were finally organised according to their intensity of binding to $PadA_{6His}$, Hsa_{His6}(+) or Hsa_{6His}(-). This was again based on the LogFC value. The highest value for the shortlisted proteins in each dataset was identified, which was a LogFC of 12.14 for PRP and 11.98 for PPP. Based on this, binding intensity of the proteins was divided into three categories: + denotes proteins with a LogFC value of 1-4; ++, with a LogFC value of 5-8; and +++, with a LogFC value of 9-12.

4.2.2 Proteins of interest identified in PRP and PPP

One group of proteins that were bound by all three His-tagged proteins in both PRP and PPP, albeit to differing levels, were components of the complement system (Tables 4.1 and 4.2). These included complement proteins C2, C3, C4-A/C4-B, C5, C6 and C8. Within PRP, it was observed that Hsa_{6His} (-) tended to demonstrate the highest binding affinity for these proteins, while in PPP, this was seen for Hsa_{6His} (+). Within PRP, low level binding of complement protein C7 was also detected but was not observed in PPP. Alongside complement proteins, interactions with regulators of the complement system were recorded. All three adhesins exhibited binding to FH in both PPP and PRP (Tables 4.1 and 4.2). Within PPP, binding to complement factor 1 and protease C1 inhibitor was also detected, with particularly high affinity binding seen for Hsa_{6His} (+).

A second group of proteins that were shortlisted were those associated with the coagulation cascade (Tables 4.1 and 4.2). Within both PRP and PPP, binding by all three adhesins was detected for prothrombin, plasminogen and coagulation factors XII and XIII. Fibrinogen (β-chain) was bound strongly by all three His-tagged

proteins in PRP, whereas in PPP, binding to fibrinogen (α-chain) was detected, particularly by Hsa_{6His} (+). Within PRP (but not PPP), binding to negative regulator of coagulation, antithrombin-III, was seen, with $PadA_{6His}$ and Hsa_{6His} (-) exhibiting comparable affinities.

Another category of proteins that was shortlisted was ECM proteins. Fibrinogen has already been mentioned above. In PRP, vitronectin was identified and bound most strongly to $PadA_{6His}$ (Table 4.1). A similar trend was seen for plasminogen, although this was detected in both PRP and PPP. Interactions with lumican, a proteoglycan that can stabilise collagen fibrils (Shao et al., 2012) was detected in PPP, with the highest affinity interaction recorded for Hsa_{6His} (+).

Finally, binding to platelet integrin receptor GPIb was detected in PPP, with the highest affinity seen with Hsa_{6His} (+) (Table 4.2).

Table 4.1 – Proteins of interest bound by Hsa or PadA in PRP

Identified protein	Associated function(s)	Binding intensity*		
		$PadA_{6His}$	**Hsa_{6His} (-)**	**Hsa_{6His} (+)**
Complement C2	Complement	+	+++	++
Complement C3	Complement	++	++	+
Complement C4-A	Complement	++	++	+
Complement C4-B	Complement	++	++	+
Complement C5	Complement	++	++	+
Complement C6	Complement	+	+++	++
Complement C7	Complement	+	+	+
Complement C8	Complement	++	++	+
Complement Factor H	Complement Regulation	++	++	+
Coagulation Factor XII	Coagulation	++	++	+
Coagulation Factor XIII	Coagulation	+	+	+
Prothrombin	Coagulation	++	++	+
Fibrinogen Beta	Coagulation / ECM protein	+++	+++	+++
Plasminogen	Coagulation/ ECM protein	+++	++	+
Heparin Co-factor 2	Coagulation	+++	+	++
Antithrombin-III	Coagulation Inhibition	++	++	+

Vitronectin	Cell Adhesion	++	+	+

*Based on the protein LogFC values: +, 1-4, ++, 5-8; +++, 9-12

Table 4.2 – Proteins of interest bound by Hsa or PadA in PPP

Identified protein	Associated function(s)	Binding intensity*		
		$PadA_{6His}$	Hsa_{6His} (-)	Hsa_{6His} (+)
Complement Factor 1	Complement Regulation	++	++	+++
Complement C2	Complement	++	++	+++
Complement C3	Complement	+	++	++
Complement C4-A	Complement	+	++	+++
Complement C4-B	Complement	+	++	+++
Complement C5	Complement	+	+	++
Complement C6	Complement	++	+	+++
Complement C8	Complement	++	++	+++
Complement Factor H	Complement Regulation	++	++	+++
Protease C1 Inhibitor	Complement Inhibition	++	++	+++
Fibrinogen-alpha chain	Coagulation / ECM protein	+	+	++
Prothrombin	Coagulation	+	+	++
Plasminogen	Coagulation / ECM protein	+	+	++
Kininogen	Coagulation	++	+	+
Coagulation Factor XI	Coagulation	$	+	$

Coagulation Factor XII	Coagulation	+	+	++
Coagulation Factor XIII	Coagulation	+	++	+++
Heparin Co-factor 2	Coagulation	+	+	++
Lumican	ECM Protein	++	++	+++
GPIb (platelet)	Platelet Adhesion	+	++	++

*Based on the protein LogFC values: +, 1-4, ++, 5-8; +++, 9-12

$, ligand interaction with the bait protein weaker than the control

4.2.3 Shortlisting of proteins identified in HCAECs

The principal aim of the work with the HCAEC lysates was to try and identify potential receptors targeted by Hsa in mediating attachment by *S. gordonii*. Consequently, $PadA_{6His}$ was not included in this analysis. Initially, 618 proteins were identified for both forms of Hsa, but as for PRP and PPP, protein lists were then further refined based on the sample hits (PSMs). Here, a value of 5 rather than 15 was used as the cut-off, as the overall PSMs values for HCAECs were generally lower than seen for plasma. Using this value to shortlist the proteins, this left 334 proteins for both forms of Hsa. Proteins with LogFC values of 0.5 or below were also excluded. This left 52 proteins remaining for both forms of Hsa. Interestingly, throughout this screening, both forms of Hsa generated the same protein number after each shortlisting check-point. Based on the LogFC range of these shortlisted proteins, which was also much lower than seen for PRP/PPP samples, binding intensity was classified as + for proteins with LogFC values of 0.5-1.0, ++ for 1.1-

1.9, and +++ for values above 2.0. The dataset was further shortlisted using UniProt, to remove proteins based on status and annotation, using the same parameters as described above for PPP and PRP. As the purpose of this analysis was to identify potential HCAEC binding receptors for Hsa, a final shortlisting was performed using UniProt based on predicted/known cellular location. All proteins associated with organelles or the nucleus were removed, as the key focus of this study was to identify potential cell-surface associated proteins that may bind to Hsa. This gave a shortlist of 13 proteins, some of which were without an identity within the dataset. Those with identified protein and gene names are listed in Table 4.3.

4.2.4 Proteins of interest identified on HCAECs

ECM protein collagen type I was identified with significant hits recorded for both collagen α-2(I) chain and collagen type I- α1 chain. These were both bound more strongly by Hsa_{6His} (+) than by Hsa_{6His} (-). A similar trend in binding was demonstrated with vimentin, an intermediate filament protein commonly associated with endothelium exposed to shear stress, such as the cardiac endothelium (Xu et al., 2004)a secretory form has been reported on the luminal surface of the blood vasculature (Xu et al., 2004).

Both keratin type I and II were strongly bound by both Hsa_{6His} (+), and Hsa_{6His} (-). Keratin provides an adaptable scaffold to epithelial/endothelial cells, allowing them to sustain various mechanical stresses. However, keratin is more commonly associated with epithelial rather than endothelial cells (Traweek et al., 1993). The presence of keratin was due to contamination, and this is recognised as a common issue with mass spectrometry (Hodge et al., 2013).

Cell junction proteins were also identified, with desmoglein-1 and ZO-2 showing high binding affinity to both forms of Hsa. These were shortlisted as similar proteins have been implicated in facilitating bacterial adhesion and subsequent colonisation (Costa et al., 2013).

Table 4.3 – Proteins of interest bound by Hsa in HCAEC lysates

Identified Protein	**Cellular Location**	**Binding intensity***	
		Hsa_{6His} (-)	**Hsa_{6His} (+)**
Collagen alpha 2(I) chain	Cytoplasm / ECM	+	++
Collagen Type 1-alpha1	Cytoplasm / ECM	+	++
Desmoglein-1	Cell Membrane	++	++
Keratin Type I	Cytoplasm/Nucleus	+++	++
Keratin Type II	Cytoplasm	++	+++
Tight Junction ZO-2	Cell Junction	++	+++
Vimentin	Cytoplasm / Secreted form	+	++

*Based on the protein LogFC values: +, 0.5-1.0; ++, 1.1-1.9; +++, above 2.0

4.3 Ingenuity Analysis Pathway

Ingenuity Pathway Analysis (IPA) is an advanced, web-based bioinformatics tool from QIAGEN, which analyses proteomics data and gene expression patterns using a scientific literature database. IPA can be utilised for various analyses, such as

toxicology or microRNA sequencing, and all data can be transferred to Microsoft Excel for ease of use. IPA provides information on detected genes, including cellular location, name and drug interactions. In addition, a main results page lists several output functions of IPA, to include canonical pathways, diseases and function, and upstream regulators (Jiménez-Marín et al., 2009; Krämer et al., 2014).

In this study, IPA was applied to the original pull-down assay proteomics data to try and identify specific interactions that were occurring at the molecular level between Hsa or PadA with proteins within PRP and PPP. Two aspects of analysis were explored in this study – canonical pathways and biological (bio) functions. Canonical pathways are pathways that demonstrate the relationship between the genes identified in a given experiment. There are usually two key pathway types identified, metabolic and signalling, which are grouped in order of activation intensity (i.e. how much these pathways are activated after protein-ligand binding occurred). Bio-functions indicate biological functions that the genes (and pathways) are associated with, in both a health and disease state. Bio-functions are usually grouped based on how much a function is activated; in this case, during or after the interaction between PRP/PPP proteins and Hsa or PadA.

4.3.1 Shortlisting of canonical pathways

Canonical pathways were recorded as intensity based on a -log p-value. The activation status of these pathways was predicted using the IPA Upstream Regulator Analysis Tool, which calculates a regulation Z-score and an overlap p-value, which is based on the number of known target genes of interest in the pathway and their subsequent gene expression, relative to a control reference within

the IPA system. The pathways were arranged in descending order (highest-lowest) of -log(p) value, with values of 5 or below discarded, leaving 17 pathways. From these 17, pathways were further refined by identifying those associated with host-pathogen interactions using the search function, so as to exclude random pathways such as those associated with host-drug interactions. This gave the pathway shortlists shown in Tables 4.4 and 4.5.

4.3.2 Predicted canonical pathways

In line with the initial proteomic analyses for both PPP and PRP, two of the pathways that gave the highest intensity values were those involved in coagulation and the complement system. A third, and indeed the most significant of the pathways identified, was the acute phase signalling response. This is a complex, early defence system that is activated by trauma, infection and inflammation. It is non-specific but is a major component of the innate immune response that aims to clear pathogens and encourage healing (Chaudhry & Babiker, 2018). For both PRP and PPP; Hsa_{6His} (-), Hsa_{6His} (+) and $PadA_{6His}$ generated comparable response levels.

The coagulation and complement system pathways were also activated comparably by Hsa_{6His} (-), Hsa_{6His} (+) and $PadA_{6His}$ in both PRP and PPP.

Two other pathways of interest identified during this analysis were the extrinsic and intrinsic prothrombin activation pathways. These pathways are associated with the clotting cascade, or haemostasis. The intrinsic pathway is the longer pathway that begins with activation of Factor XII after exposure to endothelial collagen, which occurs during tissue damage. The extrinsic pathway is the shorter pathway, which

occurs once blood vessel damage has occurred. Endothelial cells release tissue factor, which activates factor VII to factor VIIa, ultimately activating Factor X to factor Xa, which is where the extrinsic and intrinsic pathways merge (Figure 4.3) (Roy et al., 2016). The extrinsic and intrinsic pathways were activated comparably by all three adhesins for both PPP and PRP.

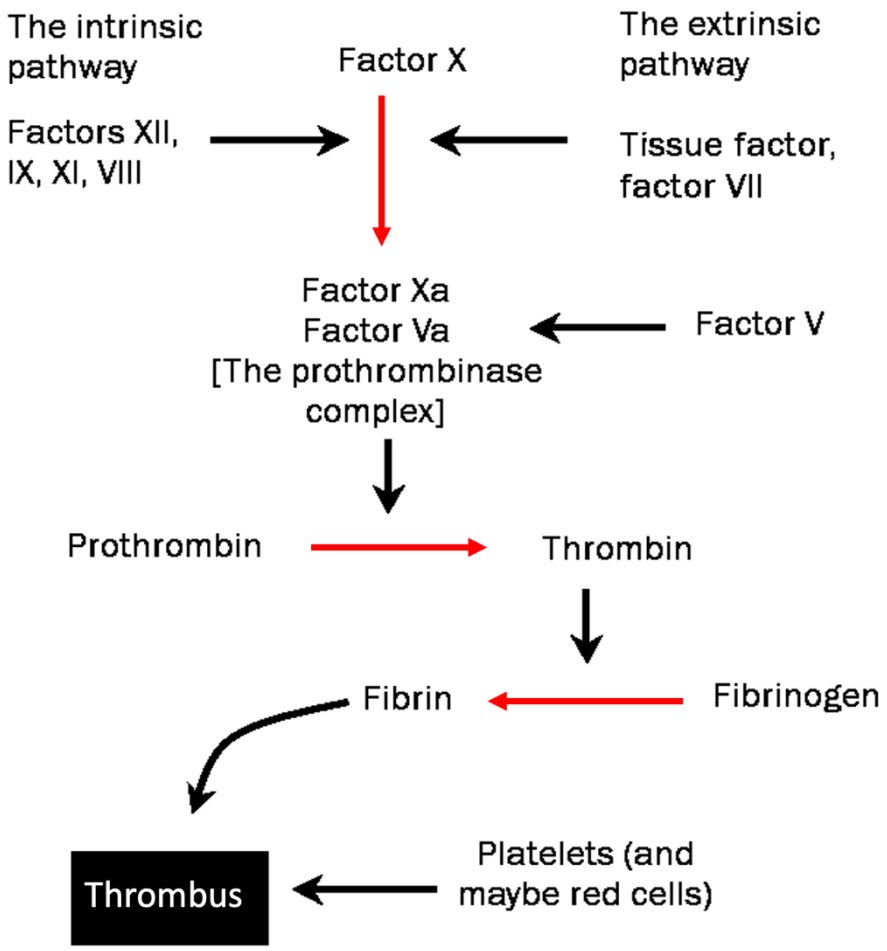


Figure 4.3 – The intrinsic and extrinsic prothrombin activation pathways, leading to the 'common pathway'. The intrinsic pathway is the longer pathway, beginning with activation of factor XII into XIIa after endothelial damage has occurred. Factor XIIa activates XI to Factor XIa, which then activates Factor IX to Factor IXa. Factor IXa subsequently activates Factor X into Factor Xa. The extrinsic pathway is activated by tissue factor, released after endothelial damage has occurred. Tissue factor activates Factor VII to Factor VIIa, which activates Factor X into Factor Xa. At this point, both pathways meet and merge, known as the common pathway. This pathway begins at Factor X, which is activated to Factor Xa. Factor Xa activates prothrombin into thrombin, which subsequently activates fibrinogen into fibrin. Thrombin is also involved, activating other factors in the intrinsic pathway (Factor XI) alongside cofactors. Fibrin subunits ultimately form fibrin strands, eventually resulting in a fibrin clot. Red arrows represent some of the key conversions associated with clot formation, of which *S. gordonii* may interact with, based on the findings within this study. Reproduced from Chaudhry & Babiker (2018) and The British Journal of Cardiology (www.bjcardio.co.uk).

Table 4.4 – Canonical pathways identified for the interactions of Hsa and PadA with PRP proteins

	Intensity (log-p)		
Pathway	**Hsa_{6His} (-)**	**Hsa_{6His} (+)**	**$PadA_{6His}$**
Acute Phase Response Signalling	47.7	47.2	46.8
Coagulation system	31.4	32.7	32.6
Complement system	26.5	27.7	25.4
Extrinsic prothrombin activation pathway	17.6	19.4	18.2
Intrinsic prothrombin activation pathway	17.6	18.8	18.4

Table 4.5 – Canonical pathways identified for the interactions of Hsa and PadA with PPP proteins

	Intensity (log-p)		
Pathway	**Hsa_{6His} (-)**	**Hsa_{6His} (+)**	**$PadA_{6His}$**
Acute Phase Response Signalling	42.7	45.9	44.7
Coagulation system	29.2	29.2	29.4
Complement system	30.8	33.0	31.0
Extrinsic prothrombin activation pathway	20.0	17.5	20.1
Intrinsic prothrombin activation pathway	19.5	17.6	17.7

4.3.3 Shortlisting of bio-functions

For all samples, an average of 502 bio-functions were initially identified. These were then divided into activated or deactivated functions. Only 2 bio-functions across all samples appeared as deactivated. These were related to cell death and removed from further consideration. For those bio-functions that were activated, bio-functions were organised according to their activation z-score. Functions with a z-score of 4 or below were disregarded, as these functions did not give a predicted activation state and appeared to be associated with host-drug interactions, rather than host-pathogen interactions. Bio-functions were then further refined based on potential relevance to bacteraemia and the pathogenesis of IE and included bio-functions associated with the immune response, host-pathogen interactions and the cardiovascular system. These shortlisted bio-functions are listed in Tables 4.6 and 4.7.

4.3.4 Predicted activation state of bio-functions

Proteins within PRP were activated in bio-functions relating to cellular movement, endothelial cell movement and activation, and cardiovascular cell movement by all 3 His-tagged adhesins. In addition, these three proteins all triggered activation of components of the inflammatory and innate immune responses, such as chemotaxis, phagocytosis, degranulation of phagocytic cells, and immune cell activation. In addition, Hsa activated functions associated with neutrophil adhesion and activation, and the activation of macrophages and APCs, although the latter was only seen for $Hsa_{6His}(+)$.

For the proteins in PPP, a generally similar profile of activated bio-functions was identified, with both Hsa and PadA associated with endothelial cell activation, chemotaxis and engagement of phagocytic cells. Again, neutrophil activation and the activation of macrophages and APCs was seen for $Hsa_{6His}(+)$. Platelet adhesion function was identified for PadA, which was not identified in PRP, but in general data on fewer functions were available for PadA in PPP than PRP.

Table 4.6 – Bio-functions and activity state identified for interactions of Hsa or PadA with PRP proteins

		Predicted activation state		
Bio Function	**Function**	**Hsa_{6His} (-)**	**Hsa_{6His} (+)**	**$PadA_{6His}$**
Cellular movement	Migration	+	+	+
Chemotaxis	Chemotaxis	+	+	+
Infectious disease	Infection of leukocyte cell lines	+	+	+
Cardiovascular system cell signalling	Endothelial cell activation	+	+	+
Cardiovascular system cell movement	Endothelial cell migration	+	+	+
Inflammatory response	Host cell immune response	+	+	+
Degranulation	Cell degranulation	+	+	+
Degranulation	Degranulation of phagocytic cells (i.e. neutrophils)	+	+	+
Cell signalling	Granulocyte activation	+	+	$
Cell-cell response	Neutrophil adhesion	+	+	$
Cell-cell response	Granulocyte adhesion	+	+	$
Synthesis	Nitric oxide synthesis	+	+	$
Inflammatory response	Phagocytosis	+	+	+
Cellular movement	Phagocyte movement	+	+	+
Cellular movement	Leukocyte movement	$	+	+
Cellular movement	Fibroblast movement	+	+	$
Cellular assembly	Fibrogenesis	+	+	$
Cellular response	T lymphocyte proliferation	$	+	$
Cellular response	Activation of antigen-presenting cells	$	+	$
Cell signalling	Macrophage activation	$	+	$
Cell maintenance	Internalisation of bacteria	+	+	$

+, binding intensity of protein ligand (activated); $, no data available

Table 4.7 – Bio-functions and activity state identified for interactions of Hsa or PadA with PPP proteins

		Predicted activation state		
Bio Function	**Function**	**Hsa_{6His} (-)**	**Hsa_{6His} (+)**	**$PadA_{6His}$**
Cellular movement	Migration	+	+	+
Cell-cell response	Platelet adhesion	$	$	+
Infectious disease	Infection of leukocyte cell lines	+	+	$
Cardiovascular system cell signalling	Endothelial cell activation	+	+	+
Cardiovascular system cell movement	Endothelial cell migration	$	+	$
Inflammatory response	Host cell immune response	+	+	+
Degranulation	Cell degranulation	+	+	$
Degranulation	Degranulation of phagocytic cells (i.e. neutrophils)	+	+	+
Cell signalling	Granulocyte activation	$	+	$
Synthesis	Nitric oxide synthesis	$	+	$
Cellular response	Organization of cytoskeleton	$	+	$
Inflammatory response	Phagocytosis	+	+	+
Cellular movement	Phagocyte movement	+	+	+
Cellular movement	Leukocyte movement	$	+	+
Cellular movement	Fibroblast movement	$	+	$
Cellular response	Respiratory burst	$	$	+
Cellular assembly	Fibrogenesis	$	+	$
Cellular response	T lymphocyte proliferation	$	+	$
Cellular response	Activation of antigen-presenting cells	$	+	$
Cell signalling	Macrophage activation	$	+	$

+, binding intensity of protein ligand (activated); $, no data available

4.4 Discussion

4.4.1 Interactive proteins identified in plasma

A major group of proteins bound by both Hsa and PadA within plasma were members of the complement system. Engagement of this immune system was further supported by the IPA analysis. Of particular note was the identification of proteins C3b and Factor H, which are associated with the opsonisation of bacterial cells. C3b labels bacterial cells for removal, whereas Factor H acts as a negative regulator of the complement system and drives the proteolytic degradation of C3b. Thus, it may be possible that engagement of Factor H by Hsa and PadA may promote complement evasion and therefore survival of *S. gordonii* within blood. A similar mechanism is seen with *S. suis,* which binds to Factor H as a cofactor in the presence of complement factor I. This leads to the degradation of C3b, thus preventing opsonophagocytosis. In addition, this recruitment enhances the capacity of *S. suis* to adhere to both epithelial and endothelial cells, although the exact mechanism is still unknown (Roy et al., 2016). The binding of Hsa and PadA to Factor H identified here may also correlate with the findings of Alves et al. (2019), for which blood isolates of *S. gordonii* showed lower levels of C3b/iC3b deposition and subsequent opsonophagocytosis relative to other strains (Alves et al., 2019). To confirm these interactions and the potential protective effects of adhesion to Factor H by *S. gordonii*, FACS could be used to quantify relative levels of surface-bound Factor H vs. C3b for wild-type *S. gordonii* and the Hsa/PadA knockout strains (Ermert et al., 2013). This technique could also be employed to confirm adhesion to the other complement proteins identified in this study to *S. gordonii*.

Another complement protein identified was C4b. This protein is derived from C4 during cleavage and release of C4a. It is involved in both the classical and lectin

pathways of the complement system, where it can also covalently attach to bacterial cell surfaces after activation (Cray et al., 2009). Protective effects via bacterial binding to C4b-binding protein, an inhibitor of the classical pathway, have been reported but not for binding C4b directly. This outcome may therefore simply be indicative of the innate immune response functioning to promote bacterial clearance via opsonophagocytosis (Cray et al., 2009)

Complement proteins C5, C6 and C8 were also bound by both Hsa and PadA. These complement proteins form the membrane attack complex (MAC), along with C7 and C9, which were not reliably identified in this study. Gram-positive bacteria such as *S. gordonii* are generally resistant to MAC killing, as the thick peptidoglycan layer of the cell wall makes it difficult for the MAC pore to form within the underlying cell membrane (Cray et al., 2009). It is possible then that binding of *S. gordonii* to these complement proteins may not significantly impact survival within the blood stream.

A general trend that was seen throughout these datasets was that Hsa_{6His} (+) appeared to bind better to the complement proteins (and most of the interactive proteins) in comparison to Hsa_{6His} (-) in PPP but not in PRP. This may indicate that Hsa preferentially targets platelets in blood, so this interaction dominates in PRP. However, when platelets are removed in PPP, binding to complement and coagulation proteins can then be detected. This aligns with a study by Deng et al. (2014), in which the selective targeting of platelet sialoglycoproteins by *S. gordonii* surface adhesins, including Hsa, was identified. This provided evidence that oral streptococci can preferentially recognise platelets, despite the availability of other sialoglycoproteins found within whole blood, with platelets then serving to deliver pathogenic streptococcal species to areas of damaged valvular endothelium during

IE pathogenesis (Liesenborghs et al., 2020). The differences in binding profiles between Hsa_{6His} (+) and Hsa_{6His} (-) in PRP and PPP also imply that glycosylation of Hsa may be important for its interactions with platelets and may enhance the efficiency of Hsa binding to target proteins in general.

Alongside complement, two other major groups of interactive proteins identified in plasma were those associated with the coagulation cascade and with the ECM. Evidence of triggering the coagulation cascade was also provided by the IPA analysis, and links with the capacity of *S. gordonii* to induce clot formation, both during vegetation development during IE and for other systemic conditions such as disseminated intravascular coagulation. Of the ECM proteins identified, the strongest binding capacity for *S. gordonii* proteins Hsa and PadA was seen with fibrinogen beta. Within PRP, vitronectin binding was also identified, most strongly with PadA. This supports the data presented as part of this project in Chapter 3 and corroborates the study of Haworth et al. (2017), in which binding of PadA to vitronectin was first proposed. It may also indicate that platelets were a major source of vitronectin, which correlates with the known capacity of *S. gordonii* to activate platelets (Cray et al., 2009)

In relation to blood survival, binding to both fibronectin and vitronectin have been shown to enable other streptococcal species to evade complement-mediated killing. *S. pyogenes* achieves complement evasion by recruiting fibrinogen to its surface, which has been proposed to prevent phagocytes from recognising deposited C3b and also inhibits the formation of the C3 convertase within the classical and lectin complement pathways (Liesenborghs et al., 2020). In addition, *S. suis*, a major contributor to septicaemia and meningitis, possesses novel fibrinogen binding proteins on its cell surface. This binding promotes antiphagocytosis by neutrophils

and also prevents complement deposition on the bacterial cell surface, thus preventing opsonisation (Hatayama et al., 2018). It is possible then that by binding fibrinogen and vitronectin, when in the blood stream, Hsa and PadA may serve to protect *S. gordonii* from complement-mediated killing (Cray et al., 2009). As already discussed, vitronectin on the bacterial cell surface prevents the association of C9 with C5-7, meaning that the MAC cannot be formed. Binding of *S. gordonii* PadA to vitronectin could also promote complement evasion via this mechanism (Liesenborghs et al., 2020).

In addition to complement evasion and overall survival within blood, the binding of *S. gordonii* via Hsa and PadA to fibronectin and vitronectin may also promote vegetation formation during IE. In native IE, this process is initiated at sites of damaged cardiac endothelium in which components of the ECM are exposed. The capacity to target fibrinogen and vitronectin may enable *S. gordonii* to attach to the damaged, roughened cardiac surface, before recruiting circulating platelets to initiate thrombosis (Hatayama et al., 2018).

4.4.2 Interactive proteins identified in HCAEC lysates

Collagen was a prominent component that was bound by Hsa within HCAEC lysates. Previous studies on *S. gordonii* adhesins SspA and SspB have indicated their ability to adhere to collagen; a mechanism that enables *S. gordonii* to invade dentinal tubules (Cray et al., 2009). Other collagen binding proteins have also been found on the surface of *S. gordonii,* such as Sgo0707, which binds to type I collagen and oral keratinocytes, and CbdA, which binds to collagen within root canals *ex vivo* (Liesenborghs et al., 2020). Nonetheless, this is first study to suggest the capacity

for Hsa to bind type I collagen (Freires et al., 2017). Cnm, expressed by another IE pathogen, *S. mutans,* is a collagen and laminin-binding protein that has been shown to readily bind to collagenous fibrils on damaged valvular tissue (Costa et al., 2013). Thus, it is possible that attachment of *S. gordonii* to collagen, including via Hsa, may represent a key mechanism that can promote vegetation development on damaged heart valves. To confirm this hypothesis, a fluorescence-based assay could be carried out, in which *S. gordonii* strains lacking Hsa are GFP-labelled and examined for their capacity to bind to purified type I collagen and to heart valves *ex vivo* (Hatayama et al., 2018). As a protein associated with collagen scaffolds, it is possible that *S. gordonii* attachment to collagen fibrils also explains why lumican was identified as an interactive protein (albeit in PPP).

A second HCAEC protein that was bound by Hsa was vimentin. Intermediate vimentin filaments are found in abundance in endothelial tissue exposed to shear stress, such as cardiac tissue (Schnittler et al., 1998). Thus, this protein might be expected to be exposed upon damage to cardiac endothelium. Furthermore, a secretory form of vimentin has been reported on the luminal surface of the blood vasculature (Xu et al., 2004). Adhesion of Hsa to vimentin may therefore represent a potential mechanism that enables *S. gordonii* to attach to both damaged and intact cardiac endothelium.

The other proteins of interest identified within the HCAEC cell lysate were those associated with endothelial cell junctions, namely tight junction protein ZO-2 and desmosome constituent desmoglein-1. Cell-cell and cell-matrix junctions are essential for maintaining polarized endothelium whilst preventing entry of pathogens (Cray et al., 2009)These junctional complexes comprise tight junctions, adherens junctions and desmosomes. However, these junctions have also become a target

for bacterial attachment and entry into the cell. For example, desmoglein-1 has previously been indicated as a key target for bacteria such as *S. aureus* to facilitate adhesion to and subsequent invasion of host cells (Sokolovska et al., 2012). Thus, Hsa may exploit similar mechanisms to mediate *S. gordonii* adherence or invasion of cardiac endothelium. To explore this in more detail, *S. gordonii* wild type and ΔHsa mutant could be tested in cell adherence or invasion assays using polarized endothelial cells with either intact or disrupted cell junctions (Liesenborghs et al., 2020), or in the presence of specific inhibitors (e.g. blocking antibodies) against ZO-2 and desmoglein-1.

In general, higher binding to HCAEC lysate proteins was seen with Hsa6His(+) compared to Hsa6His (-). This implies that glycosylation might be required for the most effective binding of Hsa to HCAEC proteins and/or that posttranslational modification is needed for correct protein conformation and therefore function. It should also be noted that direct binding to specific endothelial cell receptors was not identified, but rather to ECM, cell junction or surface-associated proteins. This correlates with the association of *S. gordonii* with native valve IE, for which the initiating factor is suggested to be the presence of a damaged or roughened endothelium for bacterial attachment and vegetation formation. Nonetheless, one disadvantage of this assay approach was the use of whole cell lysates, which meant that cytoplasmic proteins dominated the samples. It is possible that, if this assay was repeated using a cell-surface preparation of HCAECs, alternative receptors might be identified.

4.4.3 Additional pathways/responses identified

Acute phase response (APR) signalling was the pathway that generated the highest intensity via IPA analysis of plasma interactive proteins. This is a core component of the innate immune response and can be activated by a multitude of factors, such as trauma or stress (Cray et al., 2009). Another key trigger of the APR is bacterial presence, and APR proteins can help to trap microorganisms and activate the complement system (Cray et al., 2009). These data likely indicate, therefore, the induction of the host immune response upon entry of *S. gordonii* into blood. This is further supported by the identification of macrophage activation, fibroblast movement, T-lymphocyte proliferation and degranulation of phagocytic cells from the IPA analysis. Moreover, both Hsa and PadA appeared to be specific triggers of the immune response. Again, this correlates with data presented in Chapter 3, in which PadA and Hsa were implicated in neutrophil activation and induction of NETosis.

Endothelial cell activation was identified for both Hsa and PadA via IPA analysis, especially within the PRP sample. This is particularly pertinent to the pathogenesis of IE, as activated endothelial cells change from a normal anti-coagulant state into a prothrombotic state, in which they drive thrombosis via the recruitment of fibrin and platelets, and the release of tissue factor to activate the extrinsic coagulation pathway (Liesenborghs et al., 2020). Activation of fibrinogenesis is also noteworthy, as this process is associated with vegetation formation. Lesions found within IE patients are often comprised of a platelet and fibrin clot, enriched with other immune cells and bacteria (Jung et al., 2015). The potential for Hsa and PadA to induce endothelial cell activation and fibrinogenesis thus represent additional mechanisms that may enable *S. gordonii* to exacerbate infective vegetation formation.

Because the proteomic data were derived from a single pull-down assay, there were gaps in the data sets due to anomalous or unsuccessful analysis (as denoted by "$" in the relevant tables). To obtain a more complete data set and to further validate the role of Hsa and PadA in the interactions and pathways identified here, this experimental work and analysis should be repeated at least in duplicate. Furthermore, in order to explore the potential interaction networks in more depth, additional independent samples should be collected and analysed.

4.5 Summary

The overall aim of the work presented in this chapter was to perform an in-depth analysis of the proteomics data arising from previous pull-down assays to identify potential proteins within PRP, PPP and on HCAEC that may be engaged by *S. gordonii* adhesins PadA and Hsa. The key findings were as follows:

- PadA and Hsa bind strongly to complement proteins but also to proteins such as Factor H, fibrinogen and vitronectin, which may serve to protect *S. gordonii* from complement-mediated killing
- PadA and Hsa bind strongly to components of the coagulation cascade and promote endothelial cell and prothrombin activation pathways; functions that could enable *S. gordonii* to drive the unwanted thrombosis associated with IE
- Binding of Hsa to exposed ECM proteins such as collagen or lumican, or to junctional complex constituents, may facilitate *S. gordonii* attachment to damaged cardiac endothelium

- In addition to those interactions listed above, IPA analysis indicated the capacity for *S. gordonii* Hsa and PadA adhesins to induce a strong acute phase immune response, including adhesion and activation of neutrophils
- In general, glycosylation of Hsa appears to enhance its capacity to interact with host proteins

Chapter 5

Discussion

5.1 Project outline

Previous studies have demonstrated the ability of *S. gordonii* to contribute to cardiovascular disease IE (Lee et al., 2006; Mosailova et al., 2019). In particular, it has been shown that *S. gordonii* has the capacity to interact with platelets, adhering to and subsequently activating them, leading to thrombus formation on heart valves (Haworth et al., 2017a; Keane et al., 2013; Petersen et al., 2010). In addition, the ability of *S. gordonii* to adhere to components of the ECM, such as fibronectin and vitronectin (Haworth et al., 2017b; Jakubovics et al., 2009), is proposed to enable *S. gordonii* to adhere to damaged cardiac tissue. For both platelet and ECM interactions, *S. gordonii* surface adhesins PadA and Hsa have been implicated (Haworth et al., 2017b; Keane et al., 2013; Petersen et al., 2010), making them potential therapeutic targets for combatting IE. One aspect of IE pathogenesis that has been less well explored, however, is the ability of *S. gordonii* to survive within the blood stream.

To address this knowledge gap, the overall aim of this project was to investigate the interactions of *S. gordonii* with blood components and their potential implications for survival, with a particular focus on the role of adhesins PadA and Hsa. Studies were also performed to gain a better understanding of the molecular basis of *S. gordonii* interactions with cardiac endothelium. These aims were to be achieved by global analysis of essential genes (TraDIS system) and protein interactions (proteomics), combined with targeted studies using a panel of *S. gordonii* mutant strains.

5.2 *S. gordonii* survival in serum

Studies on the ability of *S. gordonii* to survive within human serum revealed the capacity of *S. gordonii* to survive over a period of 6 hours under varying conditions. In general, over this time period, bacterial numbers were reduced but not eliminated. Entry of bacteria into the blood stream would be expected to induce a rapid host immune response and indeed, evidence of this came from the IPA analysis, with significant activation of the APR indicated, alongside recruitment and activation of phagocytic cells such as neutrophils and macrophages. In vitro studies presented here also directly demonstrated the capacity for *S. gordonii* to induce neutrophil activation and NET release. As had been the original intention with this project, if performing studies with whole human blood rather than serum, the presence of phagocytic cells, for example, would be expected to significantly reduce *S. gordonii* numbers over the same time period. Supporting this, a recent study found that with a 10^4 CFU inoculum, only 5% of *S. gordonii* cells survived after 6 h in whole blood (Ha et al., 2020). Nonetheless, bacteria were still not completely cleared, supporting the hypothesis that *S. gordonii* has the capacity to protect itself against such defences. Moreover, this project has identified potential mechanisms by which *S. gordonii* may be able to achieve this, including those mediated by PadA and Hsa directly, as discussed below.

5.2.1 Interactions with the complement system

Targeting of bacterial cells by the complement system for subsequent removal by opsonophagocytosis is a major aspect of the APR, and the proteomics data presented here clearly indicated the capacity for *S. gordonii* to be bound by

complement proteins, at least in part mediated by PadA and Hsa. Nonetheless, these two adhesins also engaged proteins that may afford protection against complement-mediated killing, namely fibrinogen, Factor H and vitronectin. This may explain, at least in part, why *S. gordonii* mutants lacking these proteins were impaired in serum survival relative to wild-type.

By driving the degradation of C3b, recruitment of Factor H can inhibit C3b deposition on the bacterial cell surface, thus allowing the bacterial cell to avoid complement-mediated killing (Roy et al., 2016). Likewise, recruitment of fibrinogen can impair immune recognition of deposited C3b. This has been reported to protect a number of bacterial species, including *S. suis*, *S. pyogenes* and *S. pneumoniae* (Andre et al., 2017; Carlsson et al., 2005; Pian et al., 2015). It is possible, then, that these represent common immune evasion mechanisms amongst streptococci. However, as fibrinogen and Factor H were identified via the proteomic studies here, it will be important to verify these binding capabilities for *S. gordonii* and the potential immune protective effects. As mentioned, one way in which this could be achieved would be to monitor levels of fibrinogen or Factor H vs. C3b deposition on the bacterial cell surface by FACS following incubation of *S. gordonii* in blood or serum. This could then be correlated with survival, using both wild-type *S. gordonii* and the collection of PadA and Hsa mutant strains.

Recruitment of vitronectin to the bacterial cell surface has been widely reported to contribute to complement evasion by preventing formation of the MAC (Singh et al., 2010). Nonetheless, this mechanism has also been found to confer protection on Gram-positive bacteria such as *S. pneumoniae* (Voss et al., 2013). In this study, direct binding to vitronectin was shown for both PadA and Hsa, correlating with previous literature (Haworth et al., 2017b). It was also demonstrated that alongside

the N-terminal region (unpublished data), PadA and Hsa can target the heparin-binding site within the C-terminus of vitronectin. To further try and verify if recruiting vitronectin is protective, survival studies could be performed using vitronectin-depleted serum. Complement deposition in the presence of vitronectin could also be investigated. With the involvement of two domains within vitronectin, it would also be of interest to assess if simultaneous engagement of both sites is required for effective attachment, and if PadA and Hsa preferentially target one or other of the two domains. This could be explored, for example, by examining binding kinetics with different vitronectin domains and in the presence of specific blocking peptides.

5.3 *S. gordonii* in the progression of IE

5.3.1 Attachment to cardiac tissue

Viridans group streptococci such as *S. gordonii* have a particular association with native valve IE. As such, pathogenesis is principally linked with vegetation formation on natural heart valves that have experienced some form of damage. Bacteria (and platelets) within the blood stream are therefore presented with a roughened surface, and it is generally proposed that targeting of ECM and other exposed components initiates thrombosis. In support of this, potential targets of interest that were identified from the pull-down assay data in this study included collagen and vimentin. Using *ex vivo* pig heart valves, *S. mutans* has been shown to attach via exposed collagen fibres (Avilés-Reyes et al., 2017) and so it is possible that a similar mechanism is exploited by *S. gordonii*. Likewise, vimentin has been associated with damaged endothelium and a secretory form has been found on the luminal surface of blood vasculature (Schnittler et al., 1998; Xu et al., 2004), making it another valid

target for *S. gordonii*. Binding to vitronectin may also facilitate the ability of *S. gordonii* to form vegetations, as it is expressed in abundance on the endocardial surface (Singh et al., 2010). This was not detected in the proteomics analysis using HCAEC lysates, but this may reflect the relative abundance of protein in a whole cell lysate and/or the fact that only Hsa was used as the bait protein. To further develop this work, it would be of interest to confirm binding by *S. gordonii*, and specifically Hsa, to purified versions of these target proteins. Binding assays with damaged cardiac endothelial cells could also be performed, and differential immunolabelling of target proteins and bacteria used to demonstrate co-localisation by microscopy.

5.3.2 Interactions of *S. gordonii* with neutrophils

This work has shown that *S. gordonii* is a potent stimulator of ROS production and thus neutrophil activation. Additionally, *S. gordonii* induced NETosis, during which a neutrophil expels both chromatin and histones in an effort to 'trap' bacteria, prior to ROS release (Rosales et al., 2017). In both instances, PadA and Hsa were implicated. These findings correlate with induction of the APR upon entry of *S. gordonii* into the blood stream and would be expected to contribute to bacterial clearance. However, particularly as these effects were observed using immobilised bacteria, there are also potential implications for vegetation development. It has been suggested that activated platelets can promote neutrophil activation and that the subsequent production of NETs further contributes to the generation of vegetations on the endocardium (Lee et al., 2006a). Since it is already known that the combined effects of *S. gordonii* Hsa and PadA can activate platelets (Haworth et al., 2017; Keane et al., 2010), the stimulatory interactions of these adhesins with

neutrophils offers the potential to amplify and thus further exacerbate vegetation formation. Studies should therefore progress to exploring the interactions of *S. gordonii* wild-type and Hsa/PadA mutants with both platelets and neutrophils simultaneously.

5.3.3 Promotion of thrombosis

As described, vegetation development in IE is caused by the normal process of clot formation occurring at an unwanted site i.e. on the heart valves. Two other key aspects that were identified in this study that could enable *S. gordonii* to promote this process were triggering of the coagulation cascade and endothelial cell activation. Again, both PadA and Hsa were associated with these effects. Activated endothelial cells change from an anti-coagulant state into a prothrombotic state and promote the recruitment and activation of platelets. This ultimately leads to thrombosis and activation of the extrinsic coagulation pathway, which was also identified in this analysis (Liesenborghs et al., 2020). By stimulating these pathways, it is clear to see how *S. gordonii* at the site of endocardial damage could encourage formation of the infective vegetation. To further corroborate these data, clotting assays could be performed, in which whole human blood is flowed over immobilised *S. gordonii* ± endothelial cells in microfluidic channels (Kastrup et al., 2008). It would also be of interest to investigate the cytokine response induced in cardiac endothelial cells by *S. gordonii*. Release of proinflammatory cytokines such as IL-6 and IL-8 contribute to thrombosis and will also influence the host immune response to bacteria circulating within the blood stream (Mosevoll et al., 2018).

5.3.4 Hypothesised outcome of TraDIS

Unfortunately, because of COVID-19, the mutant library generated in this work using the adapted TraDIS protocol could not be sequenced using Illumina. However, if the visit to the Waller group had gone ahead, the aforementioned errors that occurred when preparing the library could be resurrected and subsequently undergone next generation sequencing (NGS).

Samples are prepared following the adapted protocol previously described and input DNA content is measured using qPCR. The screening process can then be carried out, whereby TraDIS sequencing primers begin sequencing within the transposon sequence. These primers provide an 8-10 base pair 'transposon tag' at the beginning of each read, which allows the user to verify that each read originates from a genuine transposon-chromosome junction. Once the first read is completed, DNA is denatured, and the transposon-specific sequencing primer can re-anneal for further reads (Barquist et al., 2016). Following sequencing, the read-out data can then be mapped against the *S. gordonii* DL1 genome. To determine whether a gene is essential or not, the number of insertions per coding region are quantified. This must be normalised for gene length, so the number of unique insertion points within the coding sequence is divided by the coding sequence length in bases. This value gives the insertion index score, which is used as a measure of essentiality (Goodall et al., 2018). Usually with TraDIS, the insertion index score frequency is bimodal – it is assumed that genes in the left mode with a low number of insertions are either essential for survival or are extremely relevant to the fitness of the bacterium. The second mode is associated with genes possessing many insertions, often deemed as non-essential (Goodall et al., 2018). Based on this, an exponential distribution model can then be fitted over the mode which includes the essential genes and

probability of each gene belonging to each mode can be calculated, with the ratio of these values termed the log likelihood score. A gene can therefore be classified as essential if its log likelihood score was less than $\log_2(12)$, based on previous protocols carried out by Goodall et al., 2018.

Once the essential genes have been mapped for *S. gordonii* mutants grown in BHY broth, this can be compared to mutants grown in blood, to identify whether any interrupted genes impacted the ability of *S. gordonii* to survive, highlighting the potential importance of these given genes in blood survival. Statistical analysis for TraDIS can be difficult, however, previous studies observed overestimation of essential genes as a key problem with this technique (Barquist et al., 2016). Some previous studies have employed a Poissonian model to determine essential genes, whereby a *P* value is derived for an insertion-free region (IFR) of a given length against the null hypothesis that, by chance, no insertions occurred in that region (Goodall et al., 2018).

Based on previous findings both within the laboratory and literature, it can be suggested that *S. gordonii* surface adhesins, such as PadA and Hsa may be essential for survival both in growth media and blood. This was highlighted in this project, whereby the removal of PadA and Hsa was shown to significantly reduce the ability of *S. gordonii* to survive in human serum, which would give a similar result to blood (Haworth et al., 2017, Nobbs et al., 2009). In addition, it can be hypothesised that stress-response genes, iron-sequestering genes and genes responsible for metabolism may be identified using TraDIS, as these gene types were noted in previous studies by Charbonneau et al., 2017 utilising a different streptococci species, *S. equi.*

In summary, further work needs to be carried out to rectify the issues identified during sample preparation, prior to sequencing. However, once these steps have been carried out, this technique will provide a useful, novel insight into the gene essentiality of *S. gordonii* which will open many doors for further research on this microorganism and its role in both bacteraemia and IE.

5.4 Future work

5.4.1 Role of additional adhesins

This work demonstrated the potential roles of both PadA and Hsa in promoting *S. gordonii* survival within blood, but additional adhesins were also implicated. For example, although the absence of both PadA and Hsa significantly reduced the ability of *S. gordonii* to bind to vitronectin, adhesion was not ablated, suggesting that other adhesins may be involved. A similar trend was seen with neutrophil interactions and ROS production. Completion of the planned TraDIS studies is one way in which other *S. gordonii* genes of importance in these studies may be identified. These genes and their role in *S. gordonii* blood survival could then be verified by generating and testing specific knockout mutants lacking these genes.

Of the *S. gordonii* adhesins described to date, another potential protein of interest is SndA. This is known to exhibit DNase activity (unpublished data) and its homologue in *S. sanguinis*, SWAN, has been shown to degrade NETs (Morita et al., 2014). Given the capacity for *S. gordonii* to induce NETosis and promote neutrophil recruitment and activation shown here, it would of interest to determine if the

enzymatic activity of SndA can afford some degree of protection against neutrophil-mediated killing.

5.4.2 Future studies with Hsa and PadA

In this study, PadA and Hsa have been shown to bind proteins that may promote immune evasion by *S. gordonii* within the blood stream,and drive infective vegetation formation via interactions with cardiac endothelium and the induction of processes such as NETosis and the coagulation cascade. Furthermore, while for some properties, such as for neutrophil activation, there was evidence of functional redundancy, for others, such as induction of NETosis and adhesion to vitronectin, the data implied co-operative function. The latter has also been observed in previous studies by Haworth et al. (2017), where it was demonstrated that Hsa is reliant on PadA expression in order to mediate biofilm formation and binding to both vitronectin and the salivary pellicle. To explore this co-dependence further, co-localisation studies could be carried out to determine whether these two surface proteins complex on the bacterial cell surface. This could be achieved by differentially tagging PadA and Hsa using fluorophores and subsequently utilising fluorescence microscopy to determine cellular location and proximity to one another (Nadrigny et al., 2006). Additionally, this study has provided supporting evidence that glycosylation might be required for effective functioning of Hsa.

Given their suggested scope of activity, these studies have further indicated that both PadA and Hsa may serve as therapeutic targets for combatting IE. Nonetheless, additional information is still required. For example, where specific ligand-receptor interactions have been identified, it will be important to further define

the specific adhesin domain(s) involved so that approaches such as peptide mimetics that block these interactions can be developed. Since current treatment for IE requires high dose, long-term antibiotics, such alternative strategies would be desirable given the ongoing crisis relating to the spread of antimicrobial resistance (Cahill et al., 2017).

5.4.3 Study limitations

Whilst this study has been valuable in advancing understanding of the ability of *S. gordonii* to survive within blood and to promote IE, *in vitro* assays are not without limitations. One major drawback of this work was the absence of conditions associated with the physiological environment, such as turbulent or shear blood flow. In order to mimic a more physiologically-relevant environment, more complex models could be used going forward in which bacteria and cardiac endothelial cells are exposed to blood under the control of an iBidi pump, which provides perfusion conditions representative of the human body (Jagau et al., 2019). Ultimately, validation of these studies using an animal model would be desirable, due to the presence of a more physiologically relevant environment, such as the rabbit aortic valve endocarditis model. With this model, vegetations can easily be identified and removed (Ge et al., 2008), and rabbit vascular physiology and immune responses are relatively close to those of a human, making it a good choice in the study of IE pathophysiology (Salgado-Pabón & Schlievert, 2016).

5.5 Conclusions

The overall aims of this work were to investigate the mechanisms by which *S. gordonii* can survive within human blood and contribute to IE pathogenesis. The abilities of wild-type *S. gordonii* and mutants lacking adhesins PadA and Hsa to interact with blood components and cardiac endothelium were extensively studied using in vitro assays, microscopic techniques and proteomics analyses. The initial focus of this project to utilise the high-throughput, next generation sequencing-based TraDIS system to identify genes required for blood survival unfortunately had to be abandoned due to several technical difficulties and ultimately the Covid-19 pandemic. Nonetheless, this instead provided the opportunity to interrogate relevant proteomics data. The culmination of this work had led to the identification of immune evasion strategies that may be exploited by *S. gordonii* to promote blood survival, to the discovery of potential receptors that may be targeted by *S. gordonii* to facilitate attachment to damaged cardiac endothelium, and to the identification of additional mechanisms, alongside platelet activation, that may allow *S. gordonii* to exacerbate the formation of infective vegetations. This information may serve as a platform from which novel therapeutic approaches for the management of IE could be developed.

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